REMARKS

I. The Invention

The subject matter of the present invention is an insoluble elastic matrix graft derived from ureter or urethra smooth muscle tissue. The present inventors established a novel method for preserving the naturally occurring acellular collagen/elastin framework in the ureter or urethra smooth muscles. The most surprising feature of the matrix is that, unlike matrices made by following the teaching of prior art, this collagen/elastin matrix retains the natural structural integrity and is therefore impermeable to urine, even when under pressure. Further, the acellular matrix is low in antigenicity, allows re-cellularization, and performs remarkably in restoring the functions of ureter or urethra. This is documented by several references submitted with this paper.

II. Status of the Claims

Claims 1-23 were originally filed and later canceled. Claims 24-28 have been added and are currently pending under examination.

III. Claim Rejections

A. Double Patenting

Claims 24-28 remained rejected under the judicially created doctrine of obviousness-type of double patenting as allegedly being unpatentable over claims 1, 3, 4, 8, and 10 of U.S. Patent No. 6,371,992. Applicants are willing to consider filing a terminal disclaimer once all pending claims are found otherwise allowable.

B. 35 U.S.C. §103

In the Final Office Action of November 4, 2004, the Examiner maintained the rejection of claims 24-28 under 35 U.S.C. §103(a) for alleged obviousness over Gregory (U.S. Patent No. 5,990,379) in light of Bishopric *et al.* (U.S. Patent No. 5,855,620) or Goldstein (U.S. Patent No. 5,632,778) or Abraham *et al.* (U.S. Patent No. 5,993,844). Applicants respectfully traverse the rejection.

Abraham et al.

Applicants first contend that the Abraham patent is not available as a prior art reference against the pending claims in the present application. In Applicants' previous response (filed July 22, 2004), it was pointed out that the present inventors had completed the subject invention prior to the effective filing date of the Abraham patent, May 8, 1997. By way of a Rule 131 declaration and a copy of the Probst *et al.* reference bearing the date stamp of May 2, 1997, Applicants sought to antedate the Abraham patent. The Examiner declined to accept the declaration and the accompanying evidence. Two reasons were provided: first, according to MPEP §716.10, a publication that is the subject of a Katz declaration cannot be relied on to antedate another reference; and second, the Probst *et al.* reference does not describe an insoluble elastic matrix graft derived from ureter or urethra smooth muscle; rather, it describes an insoluble elastic matrix derived from bladder smooth muscles.

Use of a Reference Involved in a Katz Declaration to Antedate Another Reference

Applicants respectfully disagree with the Examiner. Regarding the first reason, Applicants contend that the Examiner's interpretation of MPEP §716.10 regarding Katz declaration and antedating a cited reference is overly broad. The last paragraph of MPEP §716.10 states,

> A successful 37 C.F.R. §1.132 affidavit or declaration establishing derivation by the author, patentee, or applicant of the published application of a first reference does not enable an applicant to step into the shoes of that author, patentee, or applicant of the published application in regard to its date of publication so as to defeat a later second reference. *In re Costello*, 717 F.2d 1346, 1350, 219 USPQ 389, 392 (Fed. Cir. 1983).

Yet, a closer review of the *Costello* case (attached as Exhibit 1) reveals a fact pattern that is completely different from the present case. In *Costello*, three co-workers of the patent applicants published a paper ("the Gouldson reference") describing the invention. The authors later filed a Rule 132 declaration attesting that their paper was derived from the applicants' invention. When the applicants attempted to use the Gouldson reference as evidence of prior invention to antedate a §102(e) reference, the Cereijo reference, the USPTO rejected this

evidence and the Federal Circuit affirmed the rejection. The Court stated that the Rule 132 declaration by Gouldson *et al.* only eliminated the Gouldson reference as a prior art reference; it at best showed the applicants' conception and communication of the invention to Gouldson *et al.* Therefore, the Court concluded that the applicants did not even antedate Gouldson reference and should not be allowed to "step into Gouldson's shoes with respect to the date of publication of the Gouldson article as the date of invention." *Costello*, at 392. The Court emphasized that, "[t]he real issue is whether *all* the evidence, including the references, truly shows knowledge by another *prior to the time [Applicants] made their invention* or whether it shows the contrary." (*Costello*, at 391, emphasis in original).

In the present case, the Probst paper was not authored by four completely different people; quite to the contrary, two of the authors are named inventors on this application. Thus, it would be ludicrous to say that Applicants should not be allowed to "step into [the authors'] shoes" to use the reference as evidence of prior invention, because Applicants (the inventors) have never been outside of the authors' shoes: the inventors ***are*** the authors of the Probst reference. Unlike *Costello*, the Probst paper is not evidence that the inventors had conceived and communicated the invention to others; the Probst paper is evidence that the inventors had completed the invention by a certain date. Therefore, according to the Federal Circuit, the Probst paper does not show "knowledge by another prior to the time Applicants made their invention"; it shows the contrary. Because of these important distinctions, Applicants do not believe that *Costello* can be properly applied to the present case to prohibit the use of the Probst paper as evidence for prior invention by the inventors.

Species of the Invention

Regarding the second reason, Applicants contend that in order to properly antedate a cited reference, a prior invention evidenced in a Rule 131 declaration need not be exactly the same as the disclosure of the reference. According to MPEP §715.02,

> **[A] 37 CFR 1.131 affidavit is not insufficient merely because it does not show the identical disclosure of the reference(s) or the identical subject matter involved in the activity relied upon.** If the affidavit contains facts showing a

> completion of the invention commensurate with the extent of the invention as claimed is shown in the reference or activity, the affidavit or declaration is sufficient, whether or not it is a showing of the identical disclosure of the reference of the identical subject matter involved in the activity. (Emphasis added)

MPEP §715.03 further states:

> **Proof of prior completion of a species different from the species of the reference or activity will be sufficient to overcome a reference indirectly under 37 CFR §1.131 if the species shown in the reference or activity would have been obvious in view of the species shown to have been made by the applicant** … … Alternatively, if the applicant cannot show possession of the species of the reference of activity in this manner, the applicant may be able to antedate the reference or activity indirectly by, for example, showing prior completion of one or more species which put him or her in possession of the claimed genus prior to the reference's or activity's date. **The test is whether the species completed by applicant prior to the reference date or the activity's date provided an adequate basis for inferring that the invention has generic applicability.** (Emphasis added)

Applicants believe that these sections of the MPEP describe a situation very similar to the present case. The pending claims are directed to an insoluble elastic matrix graft derived from ureter or urethra smooth muscles. The prior invention shown by the Rule 131 declaration relates to an insoluble elastic matrix graft derived from bladder smooth muscles. Because of the obviousness-type of double patenting rejection of the pending claims over the claims of U.S. Patent No. 6,371,992 (directed to an insoluble elastic matrix derived from bladder smooth muscles), the Examiner has taken the position that the species of ureter or urethra smooth muscles is obvious over the species of bladder smooth muscles when used for preparing an insoluble elastic matrix for tissue repair. Thus, the evidence submitted along with the Rule 131 declaration demonstrates Applicants had completed an invention reasonably encompassing the species of bladder tissue and ureter or urethra tissue, even though the evidence only directly demonstrates the invention using bladder tissue.

For these reasons, Applicants believe that the Rule 131 declaration and accompanying evidence should be accepted as sufficient to antedate the Abraham patent.

Applicants hence respectfully request that the obviousness rejection based on Gregory in light of Abraham *et al.* be withdrawn.

Gregory in Light of Bishopic et al. or Goldstein et al. or Abraham et al.

Even if Abraham *et al.* is still considered a prior art reference against the pending claims, Applicants further contend that no *prima facie* obviousness has been established.

In order to establish a *prima facie* showing of obviousness, three requirements must be satisfied: all limitations of a pending claim must be expressly or impliedly disclosed by prior art references; there must be a suggestion or motivation in the art for one skilled artisan to combine the limitations; and there must be a reasonable expectation of success in making such a combination. MPEP §2143.

The pending claims are directed to a collagen/elastin matrix derived from ureter or urethra smooth muscles. By definition, a "matrix" is a cross-linking, interwoven mesh-like structure. The term "collagen/elastin matrix" refers to a combination of these two proteins present in a matrix structure. Collagen imparts strength and elastin imparts flexibility. Collagen, when present within a matrix, derives its strength to a large extent from the length of the fibers. The flexibility of elastin is due to its extensive crosslinking between polypeptides. Under a light microscope, collagen and elastin form a matrix of interwoven fibers. Under an electron microscope, both collagen and elastin individually form their own fibrous matrix. Collagen, a fibrous protein consisting of a combination of types I, II, III, *etc.*, is readily solubilized by conditions that do not appreciably solubilize elastin. Furuto *et al.* (*Methods in Enzymology* **144**:41-61, 1987, attached as Exhibit 2) describe a preferred method of solubilizing collagens, where selective extraction of collagen involves using salt and diluted acid (*see, e.g.*, pages 43-44).

On the other hand, elastin and its purification procedures are described in the review articles by Rosenbloom (attached as Exhibit 3) and Soskel *et al.* (Exhibit 4). According to Rosenbloom, the fibers of elastin are called elastic fibers, which are 90% elastin and 10% microfibrillar components. Soskel *et al.* also describe elastin as a "heavily cross-liked

meshwork, perhaps best described as a fiber rather than as a protein. It is an infinitely large polymer" (page 196).

In the present application, the claimed matrix is a collage/elastin matrix that maintains its native strength and flexibility. This means that the fiber lengths and cross-linking have not been chemically altered to weaken the integrity of the matrix. The matrix is also intact in that it can retain urine and does not have rips or tears through which urine can pass.

The Gregory reference teaches methods for elastin extraction and purification from various sources, including ureter. This reference, however, does not supply all limitations of the pending claims. Gregory describes its matrix as an "elastin matrix" rather than an "collagen/elastin matrix." As discussed above, elastin is *per se* a "matrix." There is no indication that the methods disclosed by Gregory are preserving the natural collagen/elastin matrix present in tissues such as in the ureter or urethra smooth muscles. For instance, the extraction conditions described in Gregory (column 5, lines 36-55) makes no allowance for preserving the collagen or microfibrillary components. Gregory describes the conventional methods for elastin extraction/purification, which call for acid and base treatment and/or use of heat. In fact, it is explicitly stated in EXAMPLE 3 (column 11, lines 46-58) that the elastin-based biomaterials produced according to the method taught therein "appear translucent, pearly white in color and collapsed when removed from water ***indicating the absence of collagen*** and other structurally supportive proteins" (emphasis added).

The Examiner apparently took the position that this missing limitation (a collagen/elastin matrix) can be found in the reference by Bishopric *et al.*, Goldstein, or Abraham *et al.* Bishopric *et al.* describe a generic method for producing a collagen/elastin matrix from tissues, where the descriptions generally relate to body tissues, with focus on vascular tissues such as heart valves. This is the most apparent in the examples. Similarly, the Goldstein reference describes bioprothesis derived from tissues following a decellularizing process involving enzymatic treatment. The specific tissue types discussed in this reference are skin and heart valves, although the author does not limit his invention to these tissue types. The Abraham reference teaches the production of an acellular collagenous matrix using a chemical method.

Again, Abraham *et al.* refer to the tissue types either in a general sense or name specific types that do not include ureter or urethra.

A review of the three references, however, fails to reveal any suggestion or motivation for one of skill in the art to combine the limitations found in Gregory and in the secondary references. As stated above, Gregory teaches extraction of elastin in a process far too harsh to preserve the intact collagen/elastin scaffolding. An artisan who sets out to prepare a bioprothesis described by Bishopric, Goldstein, or Abraham would find no specific suggestion or motivation in the references to combine the disclosure of Gregory, as the latter teaches the disruption of the collagenous network and therefore defeats the purpose of preparing a implantable material having required structural integrity.

Unexpected Properties of the Claimed Collagen/Elastin Matrix

Even if an artisan were somehow inspired to combine the teachings of Gregory and Bishopric, Goldstein, or Abraham, and attempted to produce a collagen/elastin matrix derived from tissues other than those actually experimented by Bishopric, Goldstein, and Abraham, there would be no reasonable expectation of success in achieving such a matrix with the properties as defined in claim 24. As far as the applicability of the disclosed methods is concerned, Bishopric, Goldstein, and Abraham appear to encompass a whole universe of tissue types. Yet, besides the certain tissue types that have been shown in the examples, these three references do not adequately address the feasibility of their methods in other tissues, particular those with special properties due to their distinct structure and functionality (*e.g.*, the properties of flexibility and water impermeability of the ureter and urethra).

There should be no dispute that there exist a large number of different types of tissues present in a human body, and that the tissues differ drastically in terms of strength, elasticity, and porosity of the cellular matrix. For instance, heart valves are a type of highly specialized tissue in that the tissue is nearly acellular except for a thin external layer of cells that can be readily removed by a variety of treatment methods to achieve a thick, dense, and relatively stiff matrix. In contrast, the intact matrix of smooth muscle such as that forms ureter

or urethra provides a waterproof sheath with much higher flexibility. The matrix taught by Bishopric, Goldstein, or Abraham cannot substitute the matrix provided by the present disclosure, because the matrix of the present invention provides some important features that cannot be predicted or expected from the teaching of the cited references: first, the matrix of the present invention must be strong enough retain fluids without leakage, even when under pressure; second, the matrix must be flexible enough to accommodate changing fluid pressure; third, the matrix can be sutured without compromising its internal integrity or tearing when under pressure; and fourth, the matrix must be able to sustain growth of multiple cell types (muscle, epithelial, mucosal, and nerve) necessary to restore function to the repaired organ. The inventors of this application have successfully accomplished the goal of making a matrix that has these properties and performed well in actual testing. This is evidenced by Sievert *et al.* (*World J. Urol.* **18**:19-25, 2000, attached as Exhibit 5). Three additional publications by the present inventors provide further details in the surprisingly outstanding quality of the ureter/urethra-derived collagen/elastin matrix that allowed successful graft and long term use. The performance of the matrix is particularly remarkable in terms of recellularization and pressure profile (Dahms *et al.*, *Urology* **50**:818-825, 1997, attached as Exhibit 6; Sievert *et al., J. Urol.* **163**:1958-1965, 2000, attached as Exhibit 7; and Sievert *et al., J. Urol.* **165**:2096-2012, 2001, attached as Exhibit 8). On the other hand, a matrix produced from any randomly selected tissue type simply cannot be expected to achieve the exceptional properties of the matrix of the present invention, which are essential for ureter or urethra repair.

In the Final Office Action mailed November 4, 2004, the Examiner sustained the obviousness rejection based on Gregory and secondary references by Bishopric, Goldstein, and Abraham, merely stating that the references all relate to "treatment of vascular tissues," which provides "the nexus between the primary and secondary references" (page 5 of the Final Office Action). Applicants disagree with this generalized assertion and respectfully request that the Examiner reconsider the rejection in light of Applicants' specific arguments as well as evidence submitted herein regarding the significant difference among various tissue types, particularly the

distinct structural and functional features of urethra or ureter that set these tissues apart from those actually tested by Bishopric, Goldstein, and Abraham.

Summary

Because the cited references provide no suggestion or motivation to combine the claim limitations that might be found in the references, and there is certainly no reasonable expectation that such combination would produce a collagen/elastin matrix having the properties of flexibility, water impermeability, and susceptibility for cell repopulation, which is essential for a graft used for repairing ureter or urethra. Applicants submit that no *prima facie* case of obviousness is established.

Furthermore, the claimed collagen/elastin matrix has been shown to have performed remarkably well in numerous experiments, particularly in terms of maintaining structural integrity of the graft, the ability to respond to pressure, and the capability to promote cell re-growth. These characteristics of the claimed graft simply cannot be expected or predicted from the prior art.

Accordingly, the withdrawal of the §103 rejection based on Gregory in light of Bishopric *et al.* or Goldstein *et al.* or Abraham *et al.* is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

Chuan Gao
Reg. No. 54,111

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Attachment (Exhibits 1-8)
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FULL TEXT OF CASES (USPQ FIRST SERIES)

In re Costello and McClean

(CA FC)
219 USPQ 389
Decided Sept. 20, 1983
No. 83-567
U.S. Court of Appeals Federal Circuit

Headnotes

PATENTS

1. Applications for patent -- Continuing (§ 15.3)
Second application that was not copending with original application and did not reference original application cannot be given same effect as original application under Section 120.

2. Patentability -- Anticipation -- Carrying date back of references (§ 51.203)

Patentability -- Anticipation -- Prior knowledge, use or sale (§ 51.223)
Applicant who is unable to secure under Section 120 effective filing date previous to prior art reference's effective date may overcome reference by evidence of prior invention; prior art reference that is not statutory bar may be overcome by two methods; most common way to "antedate" reference is to submit affidavit satisfying Rule 131 requirements; applicant may also show that relevant disclosure is description of applicant's own work; pertinent inquiry is under 35 USC 102(e); appellants can overcome reference by showing that they were in possession of their invention prior to reference's effective date; real issue is whether all evidence, including references, shows knowledge by another prior to time appellants made their invention or whether it shows contrary.

3. Affidavits -- Anticipating references (Rule 131) (§ 12.3)

Rule 131 governs whether applicant has proved date of invention "before" reference's effective date.

4. Interference -- Evidence -- Conception (§ 41.353)

Interference -- Reduction to practice -- Constructive reduction (§ 41.755)

Abandoned application, with which no subsequent application was copending, cannot be considered constructive reduction to practice; it is inoperative for any purpose, save as evidence of conception.

Particular patents -- Communication Cable Insulation

Costello and McClean, Communication Cable Having Dual Insulated Conductors, rejection of claims 1, 4-10, 12, 13, and 17-20 affirmed.

Case History and Disposition:

Appeal from Patent and Trademark Office Board of Appeals.

Application for patent of Derek Anthony Costello and Robert McClean, Serial No. 488,900, filed July 22, 1974. From decision rejections claims, applicants appeal. Affirmed.

Attorneys:

Richard L. Schwaab, Alexandria, Va., for appellant.

Gerald H. Bjorge (Joseph F. Nakamura and Fred E. McKelvey, on the brief) for appellee.

Judge:

Before Miller, Circuit Judge, Skelton, Senior Circuit Judge, and Smith, Circuit Judge.

Opinion Text

Opinion By:

Smith, Circuit Judge.

This is an appeal from the December 5, 1979, decision of the U.S. Patent and Trademark Office (PTO) Board of Appeals (board) sustaining two rejections under section 103 of claims, 1, 4-10, 12, 13, and 17-20 of application serial No. 488,900, filed

July 22, 1974. The invention relates to "foam-skin" communication cable insulation. The principal reference relied on is Cereijo, U.S. patent No. 3,914,357, filed January 4, 1973. We affirm the rejections.

I.

On April 12, 1971, appellants filed application serial No. 132,968 for "Communication Cable Having Dual Insulated Conductors" (the original application). Appellants failed to respond to an office action and the original application was abandoned on October 19, 1972. No attempt has been made to revive the original application.

Shortly thereafter, during the period December 5-7, 1972, three of appellants' co-workers at Northern Telecom Ltd. (Northern) presented a paper on foam-skin telephone cable insulation at the 21st International Wire and Cable Symposium in Atlantic City, New Jersey. [1] The authors of that paper filed with the PTO a declaration under 37 C.F.R. §1.132 [2] on June 20, 1978, stating

that the subject matter of that publication is believed to be the invention of appellants and was disclosed to the authors by appellants while all were employed at Northern.

[1] On January 4, 1973, Cereijo, et al., filed application serial No. 321,082 for "Method of Monitoring the Application of Cellular Plastic Insulation to Elongated Conductive Material." That application issued October 21, 1975, as Cereijo, U.S. patent No. 3,914,357, the principal reference. Appellants filed application serial No. 488,900 (the second application), which was accorded an effective filing date of March 9, 1973, pursuant to section 120. [3] The second application is substantially similar to the original application. The second application, however, was not copending with the original application nor does it reference the original application. [4]

Cereijo discloses but does not claim the invention that is the subject matter of the second application. Claims 1, 4-10, 12, 13, and 17-20 of the second application were rejected under 35 U.S.C.§103 in view of Cereijo, or Cereijo taken with Moody. [5]

Because the requirements of section 120 had not been satisfied, the board refused to recognize the filing of the original application as a constructive reduction to practice of the invention. The remainder of the evidence [6] was found insufficient to establish invention by appellants prior to the effective date of Cereijo. The board noted that, even if the original application was considered to establish conception of the invention, appellants failed to prove diligence.

II.

This appeal presents the question whether appellants are entitled to rely on an application, abandoned prior to the effective date of a reference, as a constructive reduction to practice to overcome that reference, where appellants later filed a substantially identical application which is not entitled under section 120 to the date of the abandoned application.

Appellants contend that the totality of the evidence establishes prior invention by them. Having eliminated Gouldson, which has an effective date prior to that of Cereijo, appellants allege that they have, ipso facto, antedated Cereijo as well. Appellants allege

that the relevant disclosure of Cereijo is actually their own invention. Finally, appellants argue that, without exception, the mere act of filing a patent application has long been considered a constructive reduction to practice.

The Solicitor contends that while the original application established prior conception of the invention, it did not operate as a constructive reduction to practice. Further, diligence has not been shown. Relying on public policy arguments based on section 120, the Solicitor asserts that appellants should derive no benefit from their abandoned application.

III.

In section 120, Congress set forth two requirements that an applicant must satisfy in order for a later filed application to be accorded the same effect as if it were filed on the same date as an earlier application by the same inventor disclosing the same invention. Those conditions are (1) copendency of the applications, and (2) reference in the later filed to the earlier filed application.

[2] Even if an applicant is unable to secure an effective filing date previous to the effective date of a prior art reference under section 120, the applicant may overcome a reference by evidence of prior invention. A prior art reference that is not a statutory bar may be overcome by two generally recognized methods. "The most common way to 'antedate' a reference is to submit an affidavit satisfying the requirements of Rule 131." [7] (Footnotes

omitted.) Rule 131, however, is only one way of overcoming a reference that is not a statutory bar. An applicant may also overcome a reference by showing that the relevant disclosure is a description of the applicant's own work. [8] The pertinent inquiry is under 35 U.S.C. §102(e). [9] Appellants can overcome a reference by showing that they were in possession of their invention prior to the effective date of the reference. "The real issue is whether *all* the evidence, including the references, truly shows knowledge by another *prior to the time appellants made their invention* or whether it shows the contrary." [10] (Emphasis in original.)

[3] Rule 131 governs whether an applicant has proved a date of invention "before" the effective date of the reference. [11] Appellants urge this court to find prior invention on the basis of evidence that does not satisfy either the substantive requirements of Rule 131 or the standard of proof required to eliminate the reference. We decline to do so for the reasons set forth below.

The effective date of Cereijo is January 4, 1973, prior to the effective filing date of the second application (March 9, 1973). Therefore, Cereijo is properly cited as prior art under section 102(e). In order to overcome Cereijo appellants must either (1) comply with the substantive requirements of Rule 131, or (2) establish that the relevant disclosure is of their own work.

A.

Rule 131 requires proof of either "reduction to practice prior to the effective date of the reference, or conception of the invention prior to the effective date of the reference coupled with due diligence from said date to a subsequent reduction to practice or to the

filing of the application." [12]

Appellants actually reduced the invention to practice in Canada. The invention has never been actually reduced to practice in the United States.

[4] Appellants' principal contention is that the filing of the later abandoned original application constitutes a constructive reduction to practice of the invention. Appellants cite no authority, nor can they, to support their argument. It has long been settled, and we continue to approve the rule, that an abandoned application, with which no subsequent application was copending, cannot be considered a constructive reduction to practice. It is inoperative for any purpose, save as evidence of conception. [13]

While the filing of the original application theoretically constituted a constructive reduction to practice at the time, the subsequent abandonment of that application also resulted in an abandonment of the benefit of that filing as a constructive reduction to practice. The filing of the original application is, however, evidence of conception of the invention. Appellants were able to reduce the invention to writing. That writing therefore constitutes documentary evidence that appellants had conceived of the invention as of the filing date. [14] As the board found, however, appellants did not establish diligence in reducing the invention to practice. Appellants do not contest that finding. Thus, the evidence is not sufficient to antedate Cereijo under Rule 131.

B.

Appellants submitted an affidavit under Rule 132 to establish that Gouldson discloses appellants' own invention. Appellants argue that, having "antedated" Gouldson which has an effective date prior to that of Cereijo, appellants have ipso facto antedated Cereijo. Appellants' proof that Gouldson discloses appellants' own work does not enable appellants to step into Gouldson's shoes with respect to the date of publication of the Gouldson article as the date of invention. Only Gouldson is eliminated as a reference by the showing that Gouldson describes appellants' invention. Appellants did not antedate Gouldson. Appellants' evidence, at best, establishes conception and communication of the invention to Gouldson, et al., prior to the date of publication. This has no relation at all to appellants' attempt to antedate the principal reference, Cereijo.

C.

Finally, appellants argue that two Bell Laboratories articles that reference Gouldson, and the fact that Cereijo discloses the invention as prior art rather than as part of his invention, establish prior invention by appellants. Appellants have submitted no affidavit or declaration to establish that the relevant disclosure of Cereijo is of appellants' invention.

It is not sufficient that the relevant disclosure is recognized as prior art. In order to sustain their claim, appellants must adduce evidence that *appellants* invented the relevant items in the disclosure. [15] Appellants have not done so.

IV.

In summary, in order to overcome a prior art reference under section 102(e) appellants must either satisfy the substantive requirements of Rule 131 or establish that

the relevant disclosure describes their own invention. In establishing prior invention to overcome the Cereijo reference, appellants cannot rely on their earlier filed abandoned application as a constructive reduction to practice of the invention. The evidence presented by appellants is not sufficient to establish invention prior to the effective date of the reference under Rule 131. Similarly unavailing is appellants' attempt to establish that the relevant disclosure of Cereijo describes appellants' own work. Thus, appellants have not established on this record that they invented the subject matter of the invention prior to the effective date of the reference. The rejections under 35 U.S.C. §103 in view of Cereijo are, therefore, proper.

Affirmed .

Footnotes

Footnote 1. E. Gouldson, M. Farago, G. Baxter, Foam-Skin, A Composite Expanded Insulation for Use in Telephone Cables, 21 Proc. Int'l Wire & Cable Symposium (1972).

Footnote 2. 37 C.F.R. §1.132 (1982) provides in pertinent part:

"§1.132 Affidavits or declarations traversing grounds of rejection.

"When any claim of an application is rejected on reference to a domestic patent which substantially shows or describes but does not claim the invention, * * * affidavits or declarations traversing these references or objections may be received."

Footnote 3. 35 U.S.C. §120 (1976) provides in pertinent part:

"§120. Benefit of earlier filing date in the United States

"An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, * * * by the same inventor shall have the same effect, as to such invention, as though filed on the date of the prior application, *if filed before* the patenting or *abandonment of* or termination of proceedings on *the first application* * * * *and if it contains* or is amended to contain *a specific reference to the earlier filed application* ." (Emphasis supplied.)

Application serial No. 339,631 was filed March 9, 1973. The second application, serial No. 488,900, filed July 22, 1974, was a continuation of that application.

Footnote 4. Thus, the second application cannot be given the same effect as the original application under section 120.

Footnote 5. Moody, U.S. patent No. 3,733,225, issued May 15, 1973.

Footnote 6. Appellants submitted several technical papers that reference Gouldson and submitted several Rule 132 declarations.

Footnote 7. In re Facius, 408 F.2d 1396, 1404, 161 USPQ 294, 300 (CCPA 1969). See also In re Ranier, 390 F.2d 771, 773-74, 156 USPQ 334, 336-37 (CCPA 1968) (anticipatory disclosure, not a statutory bar, may be removed by Rule 131 affidavit; proof need not be of prior reduction to practice of every embodiment, but mere prior conception will not suffice).

37 C.F.R. §1.131 provides in pertinent part:

"§1.131 Affidavit or declaration or prior invention to overcome cited patent or publication.

"(a) When any claim of an application is rejected on reference to a domestic patent which substantially shows or describes but does not claim the rejected invention, * * * and the applicant shall make oath or declaration as to facts showing a completion of the invention in this country before the filing date of the application on which the domestic patent issued, * * * then the patent * * * cited shall not bar the grant of a patent to the applicant, unless the date of such patent or printed publication be more than one year prior to the date on which the application was filed in this country.

"(b) The *showing of facts shall be such* , in character and weight, *as to establish reduction to practice prior to the effective date of the reference, or conception* of the invention prior to the effective date of the reference *coupled with due diligence* from said date to a subsequent reduction to practice or to the filing of the application. * * * (Emphasis supplied.)

Footnote 8. In re Land, 368 F.2d 866, 151 USPQ 621 (CCPA 1966). See also In re Mathews, 408 F.2d 1393, 161 USPQ 276 (CCPA 1969); Facius, 408 F.2d 1396, 161 USPQ 294 ; Chisum, Patents §3.08[2] (1982).

Footnote 9. 35 U.S.C. §102(e) provides:

"§102. Conditions for patentability; novelty and loss of right to patent

"A person shall be entitled to a patent unless--

"(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, * * *[.]"

Footnote 10. Land, 368 F.2d at 878, 151 USPQ at 632.

Footnote 11. Id., see also In re Frilette, 412 F.2d 269, 274-75, 162 USPQ 163, 168 (CCPA 1969) (absent proof of derivation, ordinarily a reference can be overcome only by showing prior completion of the invention under Rule 131).

Footnote 12. 37 C.F.R. §1.131(b).

Footnote 13. Carty v. Kellogg, 7 App. D.C. 542, 1896 C.D. 188 (1896). Carty involved a fact situation almost identical to the present case. Carty filed an original application April 20, 1885, which was abandoned October 21, 1887. Kellogg filed an application July 30, 1887. Subsequently, on November 17, 1887, Carty filed a second application. An interference was generated and Carty tried to rely on the abandoned application as a constructive reduction to practice in order to establish priority. The Court of Appeals for the District of Columbia stated:

"Failing in proof of actual reduction, Carty is forced to rely upon constructive reduction to practice, and, in order to antedate Kellogg, he claims it by virtue of his abandoned application of 1885. It seems to be a reasonable and well-established principle, conformed to in the practice of the Patent Office, that an abandoned application cannot be so considered. (Hien v. Pung, C.D., 1894, 92; 68 O.G., 657.) Having lapsed, it becomes inoperative for any purpose, save as evidence of the date of conception, and to that extent it has already been considered and its weight admitted." 1896 C.D. at 191.

Footnote 14. Thus, reviewed as documentary evidence, the original application is not accorded the "same effect" as if the requirements of §120 had been satisfied. The filing is not relied on as a legally operative reduction to practice, but rather, as evidence. As noted supra, §120 governs the receipt of the same benefit as if the second application

were filed on the date of the filing of the original application. Thus, no perturbation of §120 results from consideration of the filing of the original application merely as evidence of conception of the invention.
Footnote 15. See Facius, 408 F.2d at 1407, 161 USPQ at 302.

- End of Case -

A number of factors have greatly facilitated the acquisition of data on most of the collagens described above. Use of limited proteolysis with pepsin to facilitate the release of fragments of the molecules is one of these factors. Fragmentation of the molecules constitutes a serious limitation when this approach is utilized. However, it appears likely that many of the collagens for which extensive data are now available would have remained undetected were it not possible to isolate their fragments by means of limited proteolysis. An additional factor is the ability to selectively precipitate various molecular species of collagen from solution at different ionic strength.[12] Since the selective precipitation behavior of several molecular species is dependent on the nature of the solvent in which the collagens are dissolved, this approach provides a convenient method for attaining fractionation of complex mixtures often encountered in extracts of various tissues. And finally, the selective manner in which the various collagens are distributed in vertebrate tissues serves to reduce the potential complexity of mixtures in a given tissue extract and thereby facilitates the resolution of individual molecular species.

Table V indicates the distribution of the known collagens in noncartilaginous tissues versus hyaline cartilage and cartilage-like tissues. This summary underscores several potentially interesting points concerning the relationships among the various collagens. In this regard, 4 of the 11 collagens discussed here are deposited largely, if not exclusively, in hyaline cartilage or cartilage-like tissues such as the nucleous pulposus of the intervertebral disc and vitreous humor of the eye. Of the five potentially fibril-forming collagens (group 1 molecules) two are found in cartilage. Given the qualitative and quantitative relationships between group 1 molecules noted above, it would appear that type II collagen represents the

TABLE V
DISTRIBUTION OF THE KNOWN COLLAGENS BETWEEN NONCARTILAGINOUS TISSUES AND HYALINE CARTILAGE

Collagen	Noncartilaginous	Hyaline cartilage
Group 1 molecules	I	II
	III	
	V	K
Group 2 molecules	IV	
	VI	
	VII	
	VIII	
Group 3 molecules		IX (general)
		X(hypertrophic)

cartilage counterpart of types I and III collagens while type K represents the cartilage counterpart to type V. On the other hand, group 2 molecules, which do not form fibrous aggregates of the type formed by group 1 molecules, have no known counterparts in cartilage. Similar considerations apply to group 3 molecules which are apparently cartilage-specific proteins, and have no known counterparts in noncartilaginous tissues. These considerations indicate that of all the major connective tissues, hyaline cartilages and related structures represent the most specialized tissues with respect to the number of unique collagens synthesized and deposited within the tissues. In addition, the presence of representative group 1 molecules in both noncartilaginous tissues and cartilage may suggest that the cartilage counterparts of at least some group 2 molecules as well as the noncartilaginous counterparts of group 3 molecules remain to be identified.

[2] Isolation and Characterization of Collagens and Procollagens

By DONALD K. FURUTO and EDWARD J. MILLER

Introduction

The basic approaches commonly used to isolate and characterize collagens and procollagens have been the subjects of previous reviews.[1,2] These reviews are based largely on experience gained from studies on the fiber-forming collagens (group 1 collagens of the preceding chapter[3]) and to a certain extent on studies dealing with types IV and VI collagens (group 2 collagens). Topics dealt with included (1) the choice and use of appropriate tissues and cell culture systems, (2) procedures useful in the solubilization, recovery, and purification of collagens and their respective precursor forms, and (3) techniques suitable for evaluating chain composition as well as the unique chemical properties of individual chains. In the intervening time, the approaches described in these reviews have remained in constant use and have provided the methodological foundation for much of the subsequent work. Nevertheless, several refinements in more traditional approaches as well as new developments have proven valuable, particularly in dealing with some of the more recently described

[1] E. J. Miller and R. K. Rhodes this series, Vol. 82, p. 33.
[2] H. Sage and P. Bornstein, this series, Vol. 82, p. 96.
[3] E. J. Miller and S. Gay, this volume [1].

and less prevalent collagen molecular species. The present discussion is therefore designed to provide a current summary of approaches recommended for the isolation and characterization of collagens and procollagens. The following treatment utilizes the previous reviews as a point of departure and is concerned essentially with recent developments.

Sources of Collagens (Procollagens)

Table I lists the vertebrate tissues commonly used in the preparation of the collagen types to be discussed in this review. In general, these collagens are acquired from tissues from which interstitial collagens, types I, II, or III collagens, can ordinarily be obtained. The guidelines for the acquisition and processing of appropriate tissues or organs, therefore, follow standard procedures established previously.[1]

Table II lists several cell culture systems that have been used successfully for evaluating radiolabeled procollagens synthesized *in vitro* as discussed later. The basic procedures for cell culture and recovery of the indicated procollagens remain essentially the same as discussed in the previous review on types I, II, III, IV, and V procollagens.[2] Advances in the identification of cells and conditions which produce enriched amounts of these procollagens have been made.

Recovery and Characterization of Collagens (Procollagens)

Information concerning some of the more recently described collagens indicates that their deposition in the matrix is not accompanied by any

TABLE I
VERTEBRATE TISSUES USED IN THE PREPARATION OF NOVEL COLLAGENS

Tissue	Collagen types
Basement membranes	IV
EHS sarcoma	IV
Bone	V
Aorta	VI
Nuchal ligament	VI
Placental villi	VI
Placental membranes	VII
Uterus	VI
Descemet's membrane	VIII
Hyaline cartilage	IX, K
Chondrosarcoma	IX, K
Hypertrophic cartilage	X

TABLE II
CELLS USED IN THE PREPARATION OF NOVEL COLLAGENS

Cells	Collagen types
Endodermal cells	IV
Parietal yolk sac cells	IV
Fibroblasts (ligamentum nuchae, lung, and skin)	VI
Smooth muscle cells	VI
Endothelial (bovine)	VIII
Astrocytoma	VIII
Chrondrocytes	IX, K
Hypertrophying chondrocytes	X

presently detectable extracellular alteration. The distinction between procollagen and collagen is, in these cases, perhaps a semantic one. These results contrast sharply with those for group I precursors which lose substantial portions of their molecular mass before reaching their extracellular destination. The presence of substantial portions of noncollagenous regions in groups 2 and 3 collagens has had a major impact upon the manner in which these collagens are extracted, purified, and characterized. In addition, it has been emphasized that laboratory animals could be rendered lathyritic to increase the yield of group I collagens stabilized by lysyl-dependent cross-linking. At present, lathyrogens, in particular β-aminopropionitrile fumarate, are used routinely, even though it is not clear that all groups 2 and 3 collagens utilize this cross-linking pathway. Lathyrogens have found favorable usage in increasing the solubility of collagens in two transplantable tumors—an EHS sarcoma used in the isolation of type IV[4] and a rat chondrosarcoma used in the isolation of type IX[5]—and in bone for the isolation of type V collagen.[6]

Following Extraction with Neutral Salt and Dilute Acid Solvents

The preparation of native soluble collagens by salt and dilute acid extraction follows procedures established previously[1] including the addition of proteinase inhibitors—ethylenediaminetetraacetic acid, EDTA (20 m*M*), diisopropylfluorophosphate, DFP (1–5 m*M*) or phenylmethanesulfonyl fluoride, PMSF (1–5 m*M*), *N*-ethylmaleimide, NEM (2–5 m*M*),

[4] H. K. Kleinman, M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason, and G. R. Martin, *Biochemistry* **21**, 6168 (1982).
[5] V. C. Duance, S. F. Wotton, C. A. Voyle, and A. J. Bailey, *Biochem. J.* **221**, 885 (1984).
[6] D. L. Brock, J. Madri, E. F. Eikenberry, and B. Brodsky, *J. Biol. Chem.* **260**, 555 (1985).

and pepstatin or leupeptin (1 μg/ml)—in appropriate solvents and extraction at 4° to minimize proteolysis.

Following these extractions, the use of selective salt precipitation procedures under acidic conditions has been helpful in partitioning some of the recently isolated collagens. Of interest is the observation that the tissue forms of the various collagens have, in most cases, similar overall precipitation properties to their truncated counterparts following limited pepsin proteolysis.

Group 1 Molecule: Type V. A tissue form of type V collagen has been extracted with 0.5 *M* acetic acid from 3- to 4-week-old lathyritic chick bones.[6] The bones are initially decalcified by exposure to a solution of 50 m*M* Tris–50 m*M* EDTA, pH 8.0, containing proteinase inhibitors. Following decalcification, the bones are pulverized and sequentially extracted with 1.0 *M* NaCl containing 50 m*M* Tris–50 m*M* EDTA and proteinase inhibitors, pH 7.5, and with 0.5 *M* acetic acid. The acid-soluble material containing type V collagen is selectively precipitated at 1.2 *M* NaCl following the removal of type I collagen at 0.9 *M* NaCl. The inclusion of proteinase inhibitors in the acid solvent—pepstatin (1 μg/ml) and leupeptin (5 μg/ml)—did not affect the nature of collagen extracted. Characterization of the tissue form of type V collagen by polyacrylamide gel electrophoresis showed that isolated α1(V) and α2(V) chains are of significantly greater size than the same chains derived from molecules solubilized by limited pepsin proteolysis. Rotary shadowing of the acid-extracted collagen suggested that the additional molecular mass is in the form of a globular domain at one end of the molecule. Following bacterial collagenase digestion of the chains, the remnant globular sequences migrated as 18,000- and 29,000-Da polypeptides, for α1(V) and α2(V), respectively, as determined following electrophoretic separation.

Group 3 Molecules: Type IX. A disulfide-bonded collagenous protein has been purified from a transplantable chondrosarcoma grown in lathyritic rats.[5] This protein has been shown to be immunologically and biochemically related to type IX collagen.[5] The tumor is extracted in 1.0 *M* NaCl, 50 m*M* Tris, pH 7.5, containing a cocktail of proteinase inhibitors for 16 hr at 4°. The salt-insoluble residue is removed by centrifugation, and ammmonium sulfate is added to 45% of saturation. The resultant precipitate is dissolved in 0.5 *M* NaCl, 50 m*M* Tris, pH 7.5, and precipitated in steps at 2.0 and 4.0 *M* NaCl. The 4.0 *M* NaCl precipitate is redissolved in 0.5 *M* acetic acid and selectively reprecipitated in steps at 0.7, 1.2, 2.0, and 3.0 *M* NaCl. The 1.2 *M* NaCl precipitate contained most of the type IX collagen. This preparation is further purified by chromatography on DEAE-Sepharose under conditions in which the contaminating anionic proteins are retained. On reduction, the 225,000-Da macromolecule yielded major and minor components exhibiting a M_r in the range of 60,000 and 70,000 on separation in 8% polyacrylamide gels. On limited pepsin proteolysis, the isolated molecule yielded fragments comparable to those observed when type IX collagen is isolated following limited digestion of cartilaginous tissues. In addition, antibodies to the major, 100,000-Da, disulfide-bonded, pepsin-derived fragment, designated type M in previous work,[7] reacted strongly with the isolated salt-soluble molecule.

Following Extraction of Tissues with Chaotropic Solvents

Group 2 Molecules: Type IV. Type IV collagen is prepared from an Engelbreth–Holm–Swarm (EHS) sarcoma grown in C57BL mice made lathyritic by a dietary regimen of β-aminopropionitrile fumarate.[8] An insoluble basement membrane matrix is prepared by sequential extraction of the tumor with neutral salt and dilute acid solutions.[9] The majority of the type IV collagen is efficiently extracted from the salt- and acid-extracted residue with 2.0 *M* guanidine hydrochloride (or 2 *M* urea), 50 m*M* Tris–HCl, pH 7.4, containing 2 m*M* dithiothreitol.[4] Electrophoresis of the isolated type IV collagen preparation revealed the presence of high-molecular-weight components along with two chains of M_r 185,000 and 170,000, which are apparently identical in size to those reported for procollagen type IV.[10]

Type VI. Intact type VI collagen has been extracted with 6.0 *M* guanidine hydrochloride in the presence of proteolytic inhibitors from salt- or detergent-insoluble fetal and adult tissues[11–16] and with urea containing 25 m*M* dithiothreitol from detergent- and urea-insoluble residues of human placenta[17] (see Table I).

A general scheme for guanidine hydrochloride extraction of type VI collagen,[11] which includes revisions of the first published procedure,[12] is

[7] M. Shimokomaki, V. C. Duance, and A. J. Bailey, *FEBS Lett.* **121,** 51 (1980).
[8] R. W. Orkin, P. Gehron, E. B. McGoodwin, T. Valentine, and R. Swarm, *J. Exp. Med.* **145,** 204 (1977).
[9] R. Timpl, P. Bruckner, and P. Fietzek, *Eur. J. Biochem.* **95,** 225 (1979).
[10] K. Tryggason, P. Gehron-Robey, and G. R. Martin, *Biochemistry* **19,** 1284 (1980).
[11] M. A. Gibson and E. G. Cleary, *J. Biol. Chem.* **260,** 11149 (1985).
[12] M. A. Gibson and E. G. Cleary, *Biochem. Biophys. Res. Commun.* **105,** 1288 (1982).
[13] R. Jander, D. Troyer, and J. Rauterberg, *Biochemistry* **23,** 3675 (1984).
[14] K. R. Knight, S. Ayad, C. A. Shuttleworth, and M. E. Grant, *Biochem. J.* **220,** 395 (1984).
[15] S. Ayad, C. Chambers, C. A. Shuttleworth, and M. E. Grant, *Biochem. J.* **230,** 465 (1985).
[16] H. von der Mark, M. Aumailley, G. Wick, R. Fleischmajer, and R. Timpl, *Eur. J. Biochem.* **142,** 493 (1984).
[17] R. A. Heller-Harrison and W. G. Carter, *J. Biol. Chem.* **259,** 6858 (1984).

described below. This procedure includes initial washes with phosphate-buffered saline, pH 7.4 containing 1% Nonidet P-40 and with 0.6 *M* KCl buffered with phosphate. The salt- and detergent-insoluble residues are subsequently extracted with 6.0 *M* guanidine hydrochloride, 0.1 *M* Tris, pH 8.0. This extract containing most of the type VI collagen gave a major band of M_r 140,000 on polyacrylamide gel electrophoresis under reducing conditions.

Guanidine-extracted type VI collagen has been further purified by chromatography on DEAE-cellulose and on molecular sieve columns.[13–15] Nonreduced type VI has been fractionated on Sephacryl S-400 eluted with 6.0 *M* urea, 50 m*M* Tris, pH 7.8, at room temperature.[15] Type VI eluted as a protein fraction in the excluded volume of this column. When rechromatographed on DEAE-cellulose in 6.0 *M* urea, 50 m*M* Tris, pH 8.3, at 20°, the type VI eluted soon after the initiation of the linear gradient (0–250 m*M* over a total volume of 600 ml).[15] When this fraction was evaluated by electrophoresis following reduction, the purified protein yielded a major 140,000-Da band and some fainter bands of higher molecular weight. Pepsin digestion of the nonreduced type VI gave fragments similar in size to those obtained by limited proteolysis of placenta tissue.[13–15] In addition, nonreduced as well as reduced and alkylated protein reacted strongly when tested with an antibody prepared against a major pepsin-generated fragment of type VI collagen.[15]

Urea–dithiothreitol extractions have proceeded in a manner similar to guanidine hydrochloride extractions. A standard procedure for this method of extraction has been described.[17] Placental tissue is homogenized in 25 m*M* sodium borate buffer, pH 7.8, containing 0.34 *M* sucrose and proteinase inhibitors. The insoluble residue is next extracted with 2% (v/v) Empigen BB (*n*-alkylbetaine) in 50 m*M* sodium borate buffer, pH 7.8, containing 25 m*M* NaCl and protease inhibitors. Excess detergent is removed with a borate buffer wash, and the residue extracted with 8.0 *M* urea to remove other contaminants. Addition of reducing agent, 25 m*M* dithiothreitol, to the 8.0 *M* urea buffer and reextraction of the residues produced type VI collagen in high yield. When examined by gel electrophoresis, the type VI collagen isolated by urea–dithiothreitol extractions consisted largely of a polypeptide migrating with an approximate M_r of 140,000 when compared to noncollagenous standards.

The isolated protein cross-reacted with antiserum to a detergent-insoluble fibroblast matrix glycoprotein, gp140, during electrophoretic immunoblotting.[17] Likewise immunoblots using this same antiserum showed cross-reactivity with fragments of the isolated type VI collagen produced by pepsin proteolysis and cyanogen bromide cleavage.

Following Recovery from Culture Systems

Group 1 Molecules: Type K. Type K collagen precursors have been synthesized in the presence of [^{14}C]proline and isolated from 17-day chick embryo sternal chondrocytes grown in suspension culture.[18] Following cooling and homogenization the cell pellets are extracted for 24 hr at 4° in 0.5 *M* acetic acid/pepstatin containing 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanosulfonate) and carrier collagen. The resultant suspension is dialyzed initially against water and then against 0.4 *M* NaCl, 0.1 *M* Tris–HCl, pH 7.4, containing proteinase inhibitors. Following clarification by centrifugation, the cell-extracted proteins are precipitated by the addition of solid ammonium sulfate to 40% of saturation. The precipitate is dissolved in cold 30 m*M* Tris–HCl, pH 8.3, containing 2.0 *M* urea, 0.1% Triton X-100, and 2 m*M* disodium EDTA and dialyzed against this buffer. DEAE-cellulose chromatography is then performed under native conditions at 4° using batch elutions at 0.1, 0.2, and 0.5 *M* NaCl. Type K collagen is recovered in the 0.2 *M* and 0.5 *M* NaCl eluate, and the type K procollagen chains—pro-1α, pro-2α, and pro-3α—migrated on polyacrylamide gels as components in the 170,000–200,000 M_r range following reduction. Chymotrypsin digestion of this same fraction produces three non-disulfide-bonded polypeptides comparable to 1α, 2α, and 3α chains recovered following limited pepsin proteolysis of tissues.

Group 2 Molecules: Type IV. Procollagen type IV has been synthesized in the presence of β-aminopropionitrile and isolated from culture media of cell lines, such as rat parietal yolk sac cells (PYS-2)[19] and mouse endodermal cells.[20] Type IV procollagen is precipitated from culture medium at 40–50% ammonium sulfate and recovered by centrifugation. The constituent polypeptide chains of type IV collagen recovered in this manner have an M_r in the range of 170,000–180,000 as assessed by polyacrylamide gel electrophoresis.

The type IV procollagen recovered 40–50% ammonium sulfate precipitates has been purified by affinity chromatography on heparin-Sepharose[19] or ion-exchange chromatography on DEAE-cellulose[20] as described below. For affinity chromatography, the precipitate containing the type IV procollagen is dissolved in 0.4 *M* NaCl, 50 m*M* Tris–HCl, pH 7.4, and dialyzed against 50 m*M* ammonium bicarbonate, pH 7.9. During dialysis approximately 20–40% of the total procollagen type IV and 15% of the laminin, also present in the sample, precipitated. This minor frac-

[18] C. C. Clark and C. F. Richards, *Collagen Rel. Res.* **5**, 205 (1985).
[19] I. Oberbäumer, H. Wiedemann, R. Timpl, and K. Kühn, *EMBO J.* **1**, 805 (1982).
[20] H. P. Bächinger, L. I. Fessler, and J. H. Fessler, *J. Biol. Chem.* **257**, 9796 (1982).

tion of collagen, however, could be retrieved by selective solubilization of the collagen with 0.1 *M* acetic acid and is indistinguishable from its soluble counterpart. Chromatography of the ammonium bicarbonate-soluble fraction containing a majority of the type IV is achieved on heparin-Sepharose column utilizing a linear gradient from 0 to 0.4 *M* NaCl over a total volume of 600 ml. Under these conditions, procollagen type IV eluted during the first 40% of the gradient.

Alternatively, type IV procollagen is fractionated on DEAE-cellulose equilibrated with 30 m*M* Tris–HCl, pH 7.8, containing 2.0 *M* urea and 0.1% Triton X-100. Under these conditions, type IV procollagen is unretained, while contaminating acidic proteins are retained.[20]

Type VI. The precursor forms of type VI collagen are synthesized and detected in a number of fibroblast and smooth muscle cell cultures as well as cell explant cultures.[11,15,16,21,22] The presence of ascorbate (50 μg/ml) in the culture medium has been found to improve the synthesis of type VI in culture.[11] Type VI collagen is easily identified in fibroblast-conditioned medium by electrophoresis on acrylamide gels combined with immunoblotting on nitrocellulose,[11,21] by immunoprecipitation,[15] and by affinity chromatography.[21,22]

Direct analysis of culture medium from fibroblasts and smooth muscle cells by polyacrylamide gel electrophoresis combined with immunoblotting using a type VI antiserum shows a major reducible polypeptide of M_r 150,000 and two minor reducible immunoreactive bands of higher M_r, 180,000 and 250,000.[11] These latter bands may be precursors of the 150,000-Da chain.

Alternatively newly synthesized type VI collagen can be recovered from bovine ligamentum nuchae fibroblast culture medium using a double antibody precipitation technique.[15] On reduction, the immunoprecipitate contains two fucosylated components of approximate molecular weight of 140,000 and 240,000. The 140,000-Da polypeptide resembles the 6.0 *M* guanidine-extracted tissue form, while the minor 240,000-Da polypeptide is insensitive to bacterial collagenase digestion.

From medium conditioned by bovine skin fibroblasts, type VI collagen can be purified by chromatography on agarose affinity columns containing immobilized antibodies to type VI.[21] The absorbed protein following elution from the affinity column is then evaluated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and immunoblotting on nitrocellulose against anti-type VI antibody. Results showed a reducible, sharp band with an M_r of 240,000 against globular standards. When the cell layer

[21] B. Trüeb and P. Bornstein, *J. Biol. Chem.* **259,** 8597 (1984).
[22] H. Hessle and E. Engvall, *J. Biol. Chem.* **259,** 3955 (1984).

is extracted with electrophoresis sample buffer (62.5 m*M* Tris, pH 6.8, containing 2% SDS, 5% mercaptoethanol, and 10% glycerol), immunoblotting with antiserum to type VI revealed two major reducible bands with an apparent molecular weight of 215,000 and 200,000. The presence of these smaller molecular weight bands suggested that type VI collagen undergoes a stepwise conversion from a precursor molecule of even larger constituent chains. In fact, the conversion seems to involve many steps, since the type VI present in 6.0 *M* guanidine–HCl-extracted aorta and uterus, when reduced and examined by electrophoresis, is even smaller in size (M_r = 190,000 and 180,000).[21]

MRC-5 fetal lung fibroblasts have been found to be an excellent system for the metabolic labeling and isolation of type VI collagen.[22] Fibroblasts are labeled with [^{35}S]methionine, and the medium and cell extract are preabsorbed with gelatin-Sepharose. Immunoisolation is carried out by reacting 0.5 ml of hybridoma culture medium with protein A-Sepharose that had been pretreated with rabbit antiserum to mouse IgG. The monoclonal antibodies produced by the hybridoma have been shown to be antigenic to isolated peptic fragments of type VI. This activated immunosorbent is subsequently incubated with labeled culture medium or cell extract for 2–3 hr at ambient temperature or overnight at 4°. The beads are washed 4 times with 0.5 *M* NaCl, 50 m*M* Tris, pH 7.5, containing 0.1% Tween 20 and then boiled in the sample buffer for polyacrylamide gel electrophoresis (10 m*M* Tris, pH 6.8, 4% SDS, 23% glycerol, and 2 × 10^{-3}% bromophenol blue) in the presence or absence of 0.1 *M* 2-mercaptoethanol. The samples are evaluated by SDS–polyacrylamide gel electrophoresis, and the gels are stained with Coomassie blue and processed for autoradiography. Protein isolated by this procedure, when reduced, gave rise to two polypeptides corresponding to an M_r of 240,000 and 140,000.

Type VIII. Type VIII, originally termed "EC" or "endothelial collagen," has been initially prepared from endothelial cells of bovine aorta[23,24] and rabbit cornea.[25] Presently a number of different cells other than endothelial cells derived from normal and malignant tissues are known to produce this collagen.[26,27] However, recent findings have shown that type VIII is not made or detectable in all endothelial cells.[27] Isolation of type VIII collagen from bovine endothelial cells[23,24] provides the background for the isolation of this collagen as outlined below.

[23] H. Sage, P. Pritzl, and P. Bornstein, *Biochemistry* **19,** 5747 (1980).
[24] H. Sage, B. Trüeb, and P. Bornstein, *J. Biol. Chem.* **258,** 13391 (1983).
[25] P. Benya, *Renal Physiol.* **3,** 30 (1980).
[26] K. Alitalo, P. Bornstein, A. Vaheri, and H. Sage, *J. Biol. Chem.* **258,** 2653 (1983).
[27] H. Sage, G. Balian, A. M. Vogel, and P. Bornstein, *Lab. Invest.* **50,** 219 (1984).

Bovine aortic endothelial cells grown in serum-free, medium containing β-aminopropionitrile are maintained in the presence of radiolabeled proline for periods up to 24 hr, and the culture medium is decanted. To the culture medium, proteinase inhibitors are added, and the medium clarified by centrifugation. Ammonium sulfate (20% w/v) is added to the supernatant and stirred overnight. The resulting precipitate contains largely type III procollagen and is removed by centrifugation. The ammonium sulfate concentration is increased to 50% (w/v), and the resulting precipitate containing type VIII collagen is recovered by centrifugation. The precipitate is then dissolved in 6.0 *M* urea, 50 m*M* Tris–HCl, pH 8.0, containing proteinase inhibitors, PMSF and EDTA, and dialyzed against this same buffer in preparation for chromatography. DEAE-cellulose chromatography of this fraction is performed at 4° utilizing a linear gradient from 0 to 200 m*M* NaCl or a limiting concentration of 500 m*M* to elute the retained proteins. Under these conditions, molecular species of type VIII collagen composed of chains of 125,000 Da (EC2) and 100,000 Da (EC3) are unretained, while a larger molecular species composed of chains of 177,000 Da (EC1) is eluted after the initiation of the NaCl gradient.[3,23,24,26,27]

Group 3 Molecules: Type IX. Type IX collagen precursors have been synthesized in the presence of radiolabeled proline in organ cultures of chick sterna[28,29] and in isolated chondrocytes[30] from 16- to 18-day-old chick embryos in medium containing β-aminopropionitrile. In organ culture, sterna from 16-day-old chick embryos are radiolabeled and then homogenized in 1.0 *M* NaCl containing proteinase inhibitors.[28] The salt extract is dialyzed into DEAE-cellulose starting buffer (75 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.8, containing 2.0 *M* urea and 0.01% Triton X-100). After equilibrating the column with starting buffer, the proteins are applied to the column and eluted from it with a linear gradient of 0–350 m*M* NaCl over a total volume of 200 ml. Evaluation of the eluant showed that type IX collagen eluted as a highly retained fraction under these conditions and is separated from the earlier eluting type II, procollagen type II, and precursors to type K collagen. The type IX collagen isolated in this fashion migrated as a 300,000-Da band on polyacrylamide gel electrophoresis. When reduced, the molecule yielded two bands that migrated faster than an α1(II) chain, i.e., in the molecular weight range of 80,000 to 60,000.

Alternatively, the medium from 17-day-old chick embryo sternal cartilage in organ cultures is removed, proteinase inhibitors added to it, and the proteins precipitated with 30% ammonium sulfate.[29] Polyacrylamide

[28] K. von der Mark, M. van Menxel, and H. Wiedemann, *Eur. J. Biochem.* **138**, 629 (1984).
[29] P. Bruckner, R. Mayne, and L. Tuderman, *Eur. J. Biochem.* **136**, 333 (1983).

gel electrophoresis of this precipitate gave one band of high molecular weight near the top of the gel, which could be reduced to two bands migrating slightly faster than the α1(II) chain when identified by immunoblotting. These same polypeptides are recovered from the medium by immunoprecipitation with an anti-type IX, pepsin-derived fragment.[29]

In chondrocyte cultures, synthesis in the presence of collagen gels with normal serum[30] favored production of type IX collagen. Precursors to type IX collagen are precipitated from the medium by the addition of ammonium sulfate to 30% saturation.[30] Labeled medium proteins containing added carrier type I collagen are then selectively salt precipitated. Medium proteins precipitated from culture are dialyzed against 0.5 *M* acetic acid containing pepstatin at 4° and next against 0.5 *M* acetic acid containing 0.8 *M* NaCl and pepstatin. This step precipitates carrier type I and native type II and its precursors. The supernatant containing type IX collagen is subsequently precipitated from acidic solution with 2.0 *M* NaCl or is directly dialyzed against 0.5 *M* acetic acid containing pepstatin and lyophilized. The resultant type IX collagen preparation is applied to a DEAE-cellulose column in 200 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.4. Under these conditions, type II collagen and its precursors are not retained, whereas the type IX molecular species are bound and eluted with 1.0 *M* NaCl. When evaluated by SDS–polyacrylamide gel electrophoresis, the type IX migrated as polypeptides with an approximate M_r of 84,000 and 69,000.[30]

Recently, type IX collagen has been isolated utilizing standard procedures typically used in the fractionation of cartilage proteoglycan components.[31–34] Results have shown type IX collagen to be immunologically and structurally identical to proteoglycan light (PG-Lt).[31,32] Structural identity was confirmed by similar elution properties on anion exchangers and by migration of reduced products on polyacrylamide gel electrophoresis in the presence of SDS.[31] Further, immunoblots of PG-Lt with affinity-purified polyclonal antibody specific for the largest triple helical domain of type IX, designated HMW, after SDS–polyacrylamide gel electrophoresis showed identity.[31]

[30] C. M. Kielty, A. P. L. Kwan, D. F. Holmes, S. L. Schor, and M. E. Grant, *Biochem. J.* **27**, 545 (1985).
[31] L. Vaughan, K. H. Winterhalter, and P. Bruckner, *J. Biol. Chem.* **260**, 4758 (1985).
[32] P. Bruckner, L. Vaughan, and K. Winterhalter, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2608 (1985).
[33] A. Noro, K. Kimata, Y. Oike, T. Shinomura, N. Maeda, S. Yano, N. Takahashi, and S. Suzuki, *J. Biol. Chem.* **258**, 9323 (1983).
[34] T. Shinomura, K. Kimata, Y. Oike, A. Noro, N. Hirose, K. Tanabe, and S. Suzuki, *J. Biol. Chem.* **258**, 9314 (1983).

PG-Lt is prepared by guanidine extraction of sterna,[31,32] tibias, or femurs[33,34] of 17-day-old chick embryos labeled in organ culture. The procedure for the isolation of PG-Lt from chick sterna is given below.[31,32] Following 20–24 hr of incubation, the medium is decanted. The sterna are briefly disrupted by homogenization and extracted at 0° for 16–24 hr with 4.0 *M* guanidine hydrochloride, 50 m*M* Tris–HCl, pH 8.0, containing proteinase inhibitors. The crude extract is clarified by centrifugation, and the proteins precipitated with three volumes of cold ethanol containing 1.3% (w/v) potassium acetate. The precipitate is dissolved in the 4.0 *M* guanidine buffer and fractionated under denaturing conditions into heavy (PH-H) and light (PG-L) populations by rate zonal centrifugation.[34] The PG-L fraction is subsequently separated into PG-Lb (highest density fractions >1.42 g/ml) and PG-Lt (lowest density fractions <1.34 g/ml) by cesium chloride isopycnic density gradient centrifugation.[34]

PG-Lt is then purified by DEAE-cellulose chromatography.[32] Briefly, 0.2% (w/v) Triton X-100 is added to the fraction containing PG-Lt and is dialyzed against a starting buffer of 250 m*M* NaCl, 50 m*M* Tris–HCl, 2.0 *M* urea, 0.2% Triton X-100, pH 7.4. The dialyzed sample is applied to the column and eluted with a linear gradient of 250–400 m*M* NaCl buffer over 120 ml. Under these conditions, the PG-Lt macromolecule labeled with [^{14}C]glycine or [^{35}S]sulfate eluted as a major broad peak between 60 and 75% of the gradient. When these same labeled PG-Lt fractions are subjected to SDS–polyacrylamide gel electrophoresis on 5–20% gradient gels, the intact protein migrated as a single wide band with an M_r of 300,000. On reduction with 2-mercaptoethanol, the [^{14}C]glycine-labeled protein yielded three bands: a diffuse band at 115,000 Da and two sharper bands at 84,000 and 64,000 Da.

At least one of the three polypeptide chains contains chondroitin and/or dermatan sulfate as detected by chondroitinase ABC digestion accompanied by loss of [^{35}S]sulfate label.[32] In addition, type IX contains asparagine-linked carbohydrate chains, since the PG-Lt is labeled with radioactive mannose and no other glycosaminoglycans than those mentioned above are present.[32]

Type X. Hypertrophying and/or aging chondrocytes from the epiphyseal growth region of 12-day chick embryo tibiotarsus[30,35–37] or 18-day chick embryo sterna[38,39] produce type X collagen in culture. Chondrocytes can be cultured on plastic or within collagen gels.[38]. Cells are typically grown in medium supplemented with ascorbate and containing β-aminopropionitrile and labeled at selected times for 24 hr with radiolabeled proline. The culture medium is isolated, proteinase inhibitors are added, and the medium is clarified by centrifugation. Ammonium sulfate is then added to 30% of saturation to precipitate the type X collagen.[35,36] The collagen is then solubilized in phosphate-buffered saline and dialyzed against several changes of 0.5 *M* acetic acid containing 0.9 *M* NaCl to precipitate type II collagen. Subsequent dialysis of the supernatant against several changes of 0.5 *M* acetic acid containing 2.0 *M* NaCl resulted in the precipitation of type X collagen. On denaturation, electrophoresis of this fraction showed that the type X collagen consisted solely of a 59,000-Da band as measured against collagen standards.[35,36]

When native preparations containing type X are examined by electron microscopy of rotary-shadowed samples, parent molecules revealed rods with a length of 132 nm and a knob at one end.[40] In contrast, native preparations that have been digested with pepsin show only the rod-like domain. This result is consistent with the reduction in size of the 59,000-Da polypeptide to 45,000 Da following treatment with pepsin.[39] Further, the 14,000-Da, noncollagenous domain of the 59,000-Da chain is obtained by digestion with bacterial collagenase.[41] This domain contains no disulfide bonds, but it is extremely stable probably due to strong hydrophobic interactions.

When the two different forms of type X collagen are chromatographed on CM-cellulose under denaturing conditions, the 45,000-Da chain elutes in a single peak slightly before the elution position for the α2(I) chain under conditions used to separate type I collagen chains.[37] In contrast, the 59,000-Da chain elutes as a single peak in a more basic position after the α2(I) position.[37]

Following Limited Digestion with Proteinases (Proteolysis)

With Pepsin. Details for pepsin digestion of tissue for the isolation of collagen have been outlined in a previous report.[1] This procedure has been widely used in the rapid acquisition of data on the newer collagens. In conjunction with limited proteolysis, selective salt precipitation has provided a convenient method for attaining fractionation of complex collagen mixtures encountered during solubilization. This approach is particularly effective in attaining fractions enriched for certain molecular species and has allowed the detection of collagens for which limited

[35] T. M. Schmid and H. E. Conrad, *J. Biol. Chem.* **257**, 12444 (1982).
[36] T. M. Schmid and H. E. Conrad, *J. Biol. Chem.* **257**, 12451 (1982).
[37] T. M. Schmid and T. F. Linsenmayer, *J. Biol. Chem.* **258**, 9504 (1983).
[38] G. J. Gibson, S. L. Schor, and M. E. Grant, *J. Cell Biol.* **93**, 767 (1982).
[39] G. J. Gibson, B. W. Beaumont, and M. H. Flint, *J. Cell Biol.* **99**, 208 (1984).
[40] T. M. Schmid, R. Mayne, R. R. Bruns, and T. F. Linsenmayer, *J. Ultrastruct. Res.* **86**, 186 (1984).
[41] T. M. Schmid and T. F. Linsenmayer, *Biochemistry* **23**, 553 (1984).

extractability has been a limitation. Many newer collagens are selectively precipitated at higher ionic strengths than those needed by types I through III collagens, apparently because of their more hydrophilic nature. Following initial detection of the collagenous segments following limited proteolysis, subsequent procedures have concentrated on the isolation of intact collagen molecules and their constituent chains by other means (see above).

Group 1 Molecules: Type V. Human placental type V collagen containing α1(V), α2(V), and α3(V) prepared by limited pepsin digestion had been fractionated into two native molecular species, [α1(V)$_2\alpha$2(V)] and [α1(V)α2(V)α3(V)], by two new nondenaturing methods.[42] Previously these two native molecular species have been partially resolved utilizing phosphocellulose chromatography under nondenaturing conditions.[43]

Using an IEX-540 DEAE (4 × 300 mm), high-performance liquid chromatographic column, a partially purified preparation of type V separated into two retained fractions.[42] Chromatography is achieved on this column equilibrated with a 25 m*M* Tris–HCl buffer, pH 7.5, containing 2.0 *M* urea and eluted at 0.8 ml/min with a linear gradient from 0 to 100 m*M* NaCl over 60 min. The [α1(V)α2(V)α3(V)] molecules eluted after 37 min and are followed immediately by the [α1(V)$_2\alpha$2(V)] molecules eluting at 40 min.

An alternative approach utilizes selective ammonium sulfate precipitation under acidic conditions.[42] Addition of ammonium sulfate to 134 mg/ml in 0.5 *M* acetic acid causes the [α1(V)$_2\alpha$2(V)] molecules to precipitate, while the [α1(V)α2(V)α3(V)] molecules remain soluble until a concentration of 144.7 mg/ml is reached.

Type K. Type K collagen consisting of 1α, 2α, and 3α chains has been initially prepared by proteolysis of human hyaline cartilage.[44] Upon removal of the major cartilaginous collagen, type II, from the acid–pepsin supernatant, by precipitation between 0.7 and 0.9 *M* NaCl, the native type K collagen is precipitated at 1.2 *M* NaCl under conditions similar to the preparation of type V collagen.[45] Electrophoresis of type K resolves the 95,000- to 100,000-Da chains into three differently migrating bands: the 3α chain comigrating with or slightly in front of standard α1(II) chains and two slower migrating bands, an intermediate 2α chain and the slowest 1α chain.

Type K collagen chains are also well resolved by chromatography on

[42] C. Niyibizi, P. P. Fietzek, and M. van der Rest, *J. Biol. Chem.* **259**, 14170 (1984).
[43] R. K. Rhodes and E. J. Miller, *Collagen Rel. Res.* **1**, 337 (1981).
[44] R. E. Burgeson and D. W. Hollister, *Biochem. Biophys. Res. Commun.* **87**, 1124 (1979).
[45] R. E. Burgeson, F. A. El Adli, I. I. Kaitila, and D. W. Hollister, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2579 (1976).

CM-cellulose under denaturing conditions,[44] using approaches which resolve standard interstitial collagen chains.[1] Following the initiation of the gradient, the 3α chain elutes in the α1(II) chain position followed by the 1α chain and in the most basic position, the 2α chain.

Native type K collagen is also separated from native type II collagen on CM-cellulose under nondenaturing conditions.[46] In brief, chromatography is carried out by utilizing 40 m*M* sodium acetate buffer, pH 4.8, containing 2.0 *M* urea, as starting buffer. The gradient is formed from 0 to 240 m*M* over a total volume of 600 ml. Under these conditions, the native form of type K collagen is retained on CM-cellulose and elutes in a more basic position than native type II collagen.

Comparisons of the cyanogen bromide peptides from the 1α, 2α, and 3α chains with the α1(V) and α2(V) chains by CM-cellulose chromatography and by polyacrylamide gel electrophoresis showed that these chains are clearly different.[47] Characterization of the 3α chain has revealed that it is a more highly glycosylated form of α1(II).[46] Isolation and characterization of the CNBr peptide fragments from the 2α chain following gel-permeation, high-performance liquid chromatography (HPLC) have clearly shown that 2α is a unique chain with a great deal of homology to the α1(V) chain.[48]

Group 2 Molecules. Proteolysis of these collagens and type IX collagen of group 3 has led to the acquisition of relatively high yields of these collagens leading to evaluations of their constituent chains and fragments. Fragmentation, however, has been extensive due to interruptions in the helical structure providing sites for pepsin cleavage. Two practices have been efficacious in improving the yield and isolation of this class of components. First, decreased amounts of pepsin have been utilized to further limit overall proteolysis. Second, following the period of digestion, it is advantageous to precipitate the total soluble collagen pool with the highest ionic strength needed to selectively precipitate the collagen under investigation. After centrifugation, the precipitated collagen is rapidly solubilized in 1.0 *M* NaCl, 50 m*M* Tris, pH 8.0, and the solution adjusted to pH 7.5 with Tris base to reduce the residual proteolytic activity. The soluble collagen following neutralization is dialyzed against 0.5 *M* acetic acid over 3 days. Selective salt precipitation is then repeated in a stepwise manner to recover various collagen fractions. These procedures prevent unnecessary degradation of the collagens allowing higher yields of the collagens as generally larger fragments.

[46] D. K. Furuto and E. J. Miller, *Arch. Biochem. Biophys.* **226**, 604 (1983).
[47] R. E. Burgeson, P. A. Hebda, N. P. Morris, and D. W. Hollister, *J. Biol. Chem.* **257**, 7852 (1982).
[48] D. K. Furuto and E. J. Miller, *Collagen Rel. Res.* **3**, 433 (1983).

Type VII. Type VII or "long chain" (LC) collagen has been coisolated along with type V collagen from digested human amnionic membrane.[49] Enrichment of the amount of type VII collagen is attained by reducing the pepsin concentration approximately 100-fold to 50 mg/100 g of wet weight of tissue. The pepsin-solubilized collagens are precipitated from the clarified acidic supernatants by the addition of NaCl to 1.0 *M*. The precipitate is dissolved in cold 1.0 *M* NaCl, 50 m*M* Tris–HCl, pH 7.5, and the solution titrated to pH 8.6 to inactivate residual pepsin activity. The protein dissolved in the 1.0 *M* NaCl solution is reprecipitated by dialysis against low ionic strength, 10 m*M* dibasic sodium phosphate. The precipitate is resuspended in 1.0 *M* NaCl, 50 m*M* Tris, pH 7.5. The solution is raised in steps to 1.7, 2.6, and 4.0 *M* NaCl by dialysis, and the resulting precipitates are removed by centrifugation. The 4.0 *M* NaCl precipitate contained the types V and VII collagen.

Type VII is resolved from type V collagen by chromatography on CM-cellulose and subsequently by chromatography on DEAE-cellulose.[49] Type VII collagen as isolated exists as a high-molecular-weight, disulfide-bonded aggregate. Following reduction the constituent chains exhibit an M_r in the range of 170,000–180,000 on polyacrylamide gels. When the isolated collagen is digested with pepsin (10 : 1, substrate to enzyme) a second time in 0.5 *M* acetic acid for 22 hr at 4°, two fragments, P1 and P2, of an approximate M_r of 90,000 and 70,000 are obtained following denaturation and reduction.

Type VIII. There is preliminary evidence for the presence of a type VIII collagen fragment in limited pepsin extracts of Descemet's membrane.[50]

Group 3 Molecules: Type IX. Type IX collagen fragments have been isolated from a 2.0 *M* NaCl precipitate following removal of type II (0.7–0.9 *M* NaCl) and type K collagen (1.2 *M* NaCl) from pig,[7,51,52] cow,[52] and chick,[53,54] sources. Type IX collagen is initially isolated from mammalian cartilage as a major, highly soluble, pepsin-resistant fragment of 100,000 Da.[7] Present information as reviewed in the previous article[3] indicates that type IX collagen consists of three collagenous regions designated Col 1, Col 2, and Col 3 flanked by four noncollagenous regions designated NC1, NC2, NC3, and NC4. Acquisition of these data has been largely

[49] H. Bentz, N. P. Morris, L. W. Murray, L. Y. Sakai, D. W. Hollister, and R. E. Burgeson, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 3168 (1983).
[50] U. Labermeier and M. C. Kinney, *Biochem. Biophys. Res. Commun.* **116,** 619 (1983).
[51] M. Shimokomaki, V. C. Duance, and A. J. Bailey, *Biosci. Rep.* **1,** 561 (1981).
[52] S. Ayad, M. Z. Abedin, J. Weiss, and S. M. Grundy, *FEBS Lett.* **139,** 300 (1982).
[53] C. A. Reese and R. Mayne, *Biochemistry* **20,** 5443 (1981).
[54] C. A. Reese, H. Wiedemann, K. Kühn, and R. Mayne, *Biochemistry* **21,** 826 (1982).

through studies on fragments isolated from chick sternal cartilage[53,54] following limited pepsin proteolysis.

The major disulfide-bonded fragments, HMW and LMW, and a single, non-disulfide-bonded 14,000-Da polypeptide called C4 are prepared from the 2.0 *M* NaCl precipitate following denaturation and agarose molecular sieve chromatography.[53] The HMW fragment eluted as two peaks in the molecular weight range of 300,000 through 100,000 while the LMW fragment eluted as a 30,000-Da fraction.

After reduction and alkylation, LMW yielded a polypeptide fraction which eluted approximately at 11,000 Da and is purified by rechromatography on CM-cellulose.[55] This procedure resolved three unique polypeptide chains. When cleaved with trypsin and V8 protease, they could be identified as distinct chains following fractionation of resulting peptides by reversed-phase HPLC. In addition, cyanogen bromide cleavage of the three polypeptides and Sephadex G-75 gel filtration of each of the products gave corroborating information.

When the HMW fragment is reduced and alkylated, the reduction products may likewise be resolved by a series of chromatographic steps into three unique polypeptide chains.[53,54,56] Two 51,000-Da polypeptides contained in the initial agarose (BioGel A-5m) peak, C2, are subsequently resolved by reversed-phase HPLC into two distinct, 51,000-Da polypeptides redesignated C2 and C5.[56] In contrast, a 36,400-Da polypeptide, C3, isolated by agarose (BioGel A-5m) chromatography is found to be a chain, from which a 14,000-Da piece (C4) had been removed by proteolysis.[53,54] When C2, C3, and C5 are trypsin digested and compared by reversed-phase HPLC, all three chains have different cleavage patterns indicating that these fragments came from three unique chains.[56]

With Other Proteinases. The use of additional proteinases, such as bacterial collagenase and trypsin, has facilitated the evaluation of highly specialized collagenous and noncollagenous regions protected from the general proteolytic action of these enzymes.

Group 2 Molecules: Type IV. Unique structural segments of type IV collagen have been prepared by proteolytic cleavage of basement membranes. In particular, a globular domain of type IV collagen, designated NC1, has been prepared from a salt- and acid-extracted EHS tumor basement membrane matrix.[57] The insoluble residue is homogenized in 0.2 *M*

[55] R. Mayne, M. van der Rest, D. C. Weaver, and W. T. Butler, *J. Cell. Biochem.* **27,** 133 (1985).
[56] M. van der Rest, R. Mayne, Y. Ninomiya, N. G. Seidah, M. Chreiten, and B. R. Olsen, *J. Biol. Chem.* **260,** 220 (1985).
[57] S. Weber, J. Engel, H. Wiedemann, R. W. Glanville, and R. Timpl, *Eur. J. Biochem.* **139,** 401 (1984).

NaCl, 50 m*M* Tris–HCl, pH 7.4, 2 m*M* $CaCl_2$ and digested with bacterial collagenase for two sequential 24-hr periods at ambient temperature. The reaction is stopped by adding 5 m*M* EDTA to the combined supernatants. The proteins are then precipitated by adding 3.0 *M* NaCl over 4 hr at 4° and recovered by centrifugation. The precipitated proteins are dissolved in 100–300 ml of 50 m*M* Tris–HCl, pH 8.6, containing 2.0 *M* urea, and dialyzed against this buffer in preparation for chromatography. When the solution is passed through a DEAE-cellulose column, the globular NC1 domain is unretained, while other contaminating proteins are retained. The unretained NC1 fraction is dialyzed against 0.2 *M* ammonium bicarbonate and digested a second time with collagenase (24 hr at 37°) and lyophilized. On chromatography of the second collagenase digest on BioGel A-1.5m, the NC1 globule emerged as a peak of 170,000 Da. Further evaluation of the fragment demonstrated that different subunits of the globule overlap at their amino termini with short carboxyl-terminal segments derived from the triple-helical portion of the α1(IV) or α2(IV) chain. Physical data showed that the majority of the NC1 preparation consisted of hexamers representing the carboxylterminal region of six chains derived from two type IV molecules.

A collagenous structure representing the amino-terminal segment of type IV molecules, called 7S, has been prepared from mouse EHS sarcoma basement membranes, human placenta and kidney, and bovine lens capsule following initial limited pepsin or trypsin digestions and subsequent bacterial collagenase digestion.[58] The 7S structure is readily isolated in this fashion because of its resistance to bacterial collagenase digestion at 20°.

The starting material from which 7S collagen is derived is as follows. Lyophilized EHS sarcoma is prepared by homogenization of the tumor in 0.2 *M* ammonium sulfate, pH 7.9. The homogenate is next incubated in the presence of trypsin for 4 hr at room temperature. The reaction is stopped by adding N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (0.04 mg/ml), and collagenous protein is precipitated from the supernatant by adding NaCl to 2.0 *M*.

Similarly, the starting material from human kidney or bovine lens capsule is prepared by digestion with pepsin at 15°. The solubilized proteins are initially precipitated from the acid–pepsin supernatant with 1.2 *M* NaCl. The type IV collagen preparation is then reprecipitated at 2.0 *M* NaCl at pH 7.4 as described above.

For human placenta, starting material is prepared by extraction with

[58] J. Risteli, H. P. Bächinger, J. Engel, H. Furthmayr, and R. Timpl, *Eur. J. Biochem.* **108**, 239 (1980).

3% Triton X-100, 10 m*M* EDTA, pH 7.2, for 2 hr at 4°, collection by filtration through a nylon net, and exhaustive washing with water and 0.5 *M* acetic acid. The processed material is subsequently homogenized in 0.5 *M* acetic acid. To this homogenate, pepsin is added and incubated with stirring for 20 hr at 6–8°. The soluble fraction is precipitated with 1.2 *M* NaCl. The precipitate is dissolved in 0.1 *M* acetic acid and dialyzed against 0.2 *M* NaCl, 50 m*M* Tris, pH 7.4, to remove noncollagenous contaminants and some type V. The collagenous protein is then precipitated from the supernatant by raising the NaCl concentration to 2.0 *M*.

The above collagen preparations are then passed over DEAE-cellulose (50 m*M* Tris, pH 8.6, containing 2.0 *M* urea) to remove noncollagenous contaminants which are retained under these conditions. The collagenous protein which is unretained by the column is dialyzed against 10 m*M* acetic acid and lyophilized. The material is dissolved in 0.2 *M* NaCl 2 m*M* $CaCl_2$, 50 m*M* Tris–HCl, pH 7.4, and digested with bacterial collagenase (enzyme to substrate ratio of 1 : 100). The digest is dialyzed against 0.1 *M* acetic acid and lyophilized. On molecular sieve chromatography on a BioGel A-5m column, the 7S material elutes at approximately 285,000 Da. When rechromatographed on CM-cellulose equilibrated with 1 m*M* sodium acetate, pH 4.0, containing 4.0 *M* urea, the 7S collagen elutes as a retained peak at 60–80% of a linear gradient between 0 and 0.2 *M* NaCl over 600 ml.

Purification of Collagen (Procollagen)

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) has been introduced recently as a sensitive and rapid means of separating collagen molecules, chains, and peptide cleavage products. Two main approaches have been utilized. These are gel-permeation HPLC and reversed-phase HPLC in low molarity UV-compatible solvents.

Group I Molecules. Separation of monomer, dimer, and trimer components of denatured type I collagen can be obtained by gel-permeation HPLC utilizing a TSK-4000-SW column equilibrated with 150 m*M* NaCl, 50 m*M* phosphate buffer, pH 6.5.[59] Type I collagen is dissolved in 100 m*M* acetic acid, heated for 3 min at 60°, and applied to the column.

Reversed-phase systems utilizing organic eluents, acetonitrile and tetrahydrofuran, and perfluorinated carboxylic acid counterions (trifluoroacetic and heptafluorobutyric acid) have been developed for the sep-

[59] M. van der Rest and P. Fietzek, *Eur. J. Biochem.* **125**, 491 (1982).

aration of type I procollagen chains,[60] α chains of types I, II, and III,[59,61–63] and CNBr peptides of types I, II, and III collagen as well as tryptic peptides of type I collagen.[59,64] These systems have been advantageous, because they allow detection of the peptide bond in the low ultraviolet range and use low-molarity volatile solvents to facilitate postcolumn analysis.

Utilizing the above advances, reversed-phase HPLC can be carried out with a number of commercially available silica-based supports and solvent conditions. Early studies utilized a 30-nm pore C_8 or C_{18} Vydac TP 201 (4.5 × 250 mm) column for the separation of large collagen chain components[59,60] and cyanogen bromide-cleaved peptides.[64] Human procollagen chains have been separated on the above columns using an acetonitrile-aqueous gradient containing 9 m*M* trifluoroacetic acid.[60] The best resolution of the pro-α1(I) and pro-α2(I) chains is obtained with a 28–44% acetonitrile gradient over 1 hr with an elution rate of 0.5 ml/min at room temperature. Under these column conditions, the α1(I), α2(I), pN-α1(I), and pN-α2(I) chains elute in a single fraction early in the gradient. In contrast, the procollagen chains elute much later with the pro-α1(I) chain and a small amount of pC-α1(I) eluting first and then the pro-α2(I) and a small amount of pC-α2(I) eluting later. In a further application, native type I collagen is dissolved in 10 m*M* heptafluorobutyric acid (6 mg/ml) at room temperature and heated for 2 min at 48° to denature. Samples of 100 μl are injected. The components are optimally separated using a gradient of acetonitrile between 24 and 48% in 10 m*M* heptafluorobutyric acid at a flow rate of 1.0 ml/min over 60 min.[59] Under these conditions, the α1(I) chain elutes first, followed by the α2(I) chain, the β11, the β12, and the γ components.

It should be noted, however, that current procedures do not yet allow the complete resolution of all components in a denatured collagen preparation. For instance, in another study,[62] it has been shown that one can obtain effective separations on reversed-phase HPLC between α1(I) and α2(I) chains, while α1(II) and the α1(III) trimer elute immediately after the α1(I), and the α1(III) monomer elutes prior to the α1(I). These differences are sufficient to prepare these individual chains in high purity, but

[60] M. van der Rest, C. A. Stolle, D. J. Prockop, and P. P. Fietzek, *Collagen Rel. Res.* **2**, 281 (1982).
[61] K. A. Smolenski, A. Fallon, N. D. Light, and A. J. Bailey, *Biosci. Rep.* **3**, 93 (1983).
[62] S. J. M. Skinner, B. Grego, M. T. W. Hearn, and G. C. Liggins, *J. Chromatogr.* **308**, 111 (1984).
[63] K. A. Smolenski, A. Fallon, and N. Light, *J. Chromatogr.* **289**, 29 (1984).
[64] M. van der Rest, H. P. Bennett, S. Solomon, and F. H. Glorieux, *Biochem. J.* **191**, 253 (1980).

reliable quantitative separations can be obtained only when the dimeric and trimeric components are previously removed by prior chromatography or purification. Indeed other collagen chains will be purified by reversed-phase HPLC following preliminary chromatographic separations to simplify the complex mixtures from which they are derived.

For evaluation of types I, II, and III collagen cyanogen bromide peptides and tryptic peptides, reversed-phase HPLC has provided a rapid, quantitative, and sensitive means of separating these fragments. The use of volatile solvents has facilitated the processing and recovery of separated peptides. The results have shown that resolution is particularly good for low-molecular-weight peptides, and the reproducibility is remarkable. The optimal conditions for the separation of cyanogen bromide peptides require 10 m*M* heptafluorobutyric acid as the counterion and a linear gradient between 12.8 and 44.8% acetonitrile in water over 1 hr at a flow rate of 1 ml/min.[64]

For the separation of tryptic peptides, amounts as small as 250 μg of peptides, such as α1(I)CB3, digested with trypsin, are resolved on the above reversed-phase column utilizing a 5–40% acetonitrile gradient in 10 m*M* heptafluorobutyric acid.[59] Under these conditions, the chromatogram demonstrates a number of well-resolved peaks from which purified fragments are obtained.

A gel-permeation HPLC procedure utilizing two tandem TSK-250 columns has been developed for the evaluation of cyanogen bromide cleavage products derived from collagen α chains.[65] Using a guanidine buffer, this system permits unequivocal identification of various chains, such as α1(I), α1(II), α1(III), α1(V), α2(V), and the 2α,[48] by inspection of the peptide elution patterns following a single run requiring somewhat less than 40 min.

[65] E. J. Miller, R. K. Rhodes, and D. K. Furuto, *Collagen Rel. Res.* **3**, 79 (1983).

[3] Structure and Expression of Collagen Genes

By BENOIT DE CROMBRUGGHE and AZRIEL SCHMIDT

This article will discuss the rationale for using certain methods in studying the structure of collagen genes and their expression. A few of the techniques are outlined in greater detail.

[9] Elastin: An Overview

By JOEL ROSENBLOOM

There is an obvious need within the vertebrate body for pliant materials that can stretch, twist, and bend with normal movements, as well as serve certain specialized functions. Thus, the vertebrate body is encased in a deformable skin and contains large arteries and lung tissue that possess the property of elastic recoil. The elastic properties of these tissues are due in large part to the presence of elastic fibers in the extracellular matrix. While the elastic fibers may quantitatively compose only a relatively small but important proportion of the total weight of some tissues such as the skin, in others such as the large arteries and certain specialized ligaments they are major components and may comprise greater than 50% of the dry weight. In the light microscope, the elastic fibers are highly refractive and have been identified by characteristic staining reactions, chiefly the orcein stain and reaction with resorcin–fuchsin, tetraphenyl porphonate, or other multicomponent stains.[1] While these stains are relatively specific for elastic fibers, they do not provide unequivocal identification and ultrastructural analysis may be necessary.[2] Electronmicroscopic examination of elastic fibers has revealed that they are composed of two morphologically distinguishable components[3–5] (Fig. 1). The amorphous component, so named because it usually does not possess any regular repeating structure or banding pattern, is quantitatively the largest portion comprising upward of 90% of the mature fiber. The microfibrillar component, as its name implies, is found in small fibrils roughly 10–12 nm in diameter, located primarily around the periphery of the amorphous component but, to some extent, interspersed within it. It is now clear that these two components, in addition to being distinguishable morphologically, are chemically distinct. The name elastin has been reserved for the protein which makes up the amorphous portion of the elastic fiber and which is responsible for the elastic properties. Early work on the histochemistry and chemical and physical properties of elastin has been reviewed by Partridge.[6] Other work on structure and biosynthesis is contained in the proceedings of a conference held in 1976.[7] The involvement of elastin in blood vessel disease has been discussed by Rucker and Tinker.[8]

[1] R. D. Lillie, "Histopathologic Technique and Practical Histochemistry," 3rd Ed., p. 551. McGraw-Hill (Blakiston), New York, 1965.
[2] E. N. Albert and E. Fleischer, *J. Histochem.* **18,** 697 (1970).
[3] W. H. Fahrenbach, L. B. Sandberg, and E. G. Cleary, *Anat. Rec.* **155,** 563 (1966).
[4] T. K. Greenlee, Jr., R. Ross, and J. L. Hartman, *J. Cell Biol.* **30,** 59 (1966).
[5] H. E. Darrer and J. Cox, *J. Ultrastruct. Res.* **4,** 420 (1961).

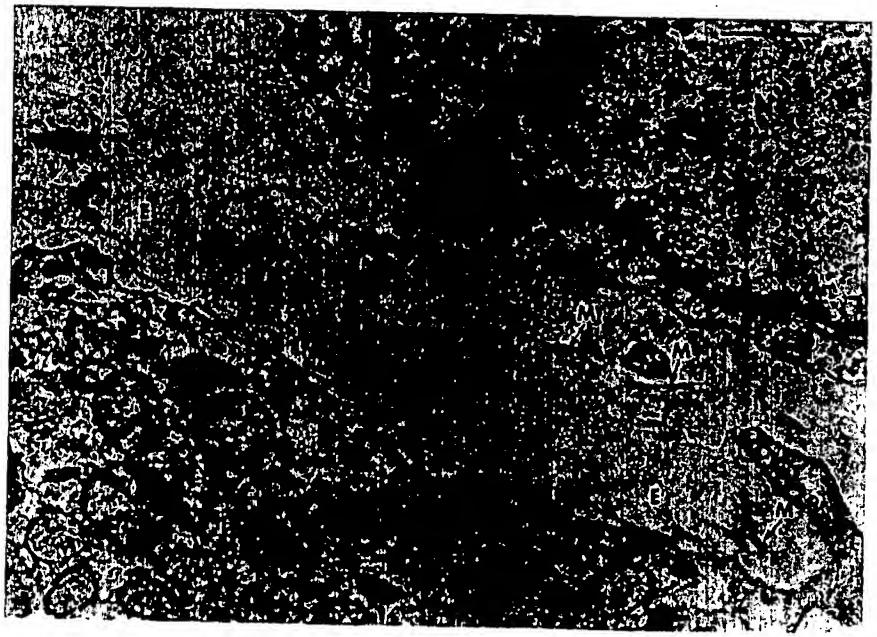

FIG. 1. Electron micrograph of 15-day chick embryo aorta. A forming elastic fiber is situated between two cells, the nucleus (N) of one of which is marked and the endoplasmic reticulum (ER) of the other. Note the microfibrils (M) on the surface of the amorphous elastin (E). Some microfibrils are interspersed within the amorphous elastin. Stained with uranyl acetate and lead citrate. ×45,000. Bar = 0.5 μm.

Isolation of Elastin

The purification of elastin has relied largely on its great insolubility under all conditions in which there is no appreciable cleavage of peptide bonds. Early methods employed relatively nonspecific and fairly harsh extraction conditions. For example, tissues were extracted with 0.1 *M*

[6] S. M. Partridge, *in* "Advances in Protein Chemistry" (C.B. Anfinsen, Jr., M. L. Anson, K. J. Bailey, and J. T. Edsall, eds.), p. 227. Academic Press, New York, 1962.
[7] L. B. Sandberg, W. R. Gray, and C. Franzblau, eds., "Elastin and Elastic Tissue" Plenum, New York, 1977.
[8] R. B. Rucker and D. Tinker, *Int. Rev. Exp. Pathol.* **17,** 1 (1977).

NaOH at 98° for 30 to 60 min or repeatedly autoclaved in water until no further protein was solubilized and the insoluble residue was operationally taken to be elastin.[9] These procedures are reasonably satisfactory for those tissues which contain fairly large amounts of elastin, such as major arteries and the ligamentum nuchae of herbivores, in that they result in preparations with a characteristic amino acid composition and appearance in the electron microscope. In cases where the elastin content is small, such as the parenchyma of fetal lungs, these methods of isolation are less satisfactory because the insoluble fraction is contaminated by other proteins. In addition to this limitation, the vigorous extraction procedures result in elastin preparations in which there is significant cleavage of peptide bonds, possible alterations in cross-link amino acids, and destruction of the microfibrillar component. ~~H.~~ Reiser page 182.

Milder methods have been developed which yield preparations in which the elastin chains are largely intact and which, in addition, yield a protein fraction at least partially derived from the microfibrillar component. The first of these procedures involves an extraction of the tissue with 5 *M* guanidine which removes the soluble collagen, glycoproteins, and proteoglycans, followed by digestion of the remaining contaminating insoluble collagen with highly purified clostridial collagenase.[10] Electron microscopic observation of the preparation at this stage reveals elastic fibers containing amorphous and microfibrillar components while no collagen or other proteins are visualized. A second extraction with 5 *M* guanidine containing 2-mercaptoethanol then solubilizes the microfibrils from the periphery of the fibers leaving an insoluble elastin residue. Amino acid analyses of the soluble fraction are rich in acidic and other hydrophilic amino acids as well as unusually rich in cystine/cysteine. Although the microfibrillar component undoubtedly contributes to the protein in this soluble fraction, subsequent electrophoretic analyses have demonstrated that this fraction is a complex mixture of proteins and glycoproteins (for review, see Ref. 11). At present it has not been determined which of these proteins forms the microfibrillar component. The amino acid composition of insoluble ligamentum nuchae elastin prepared in this fashion is similar to that prepared by hot alkali extraction. However, this procedure may not be sufficient to remove all contaminating proteins and glycoproteins from tissues containing smaller proportions of elastin, and additional steps have been added in other purification schemes.[12] These

[9] A. I. Lansing, T. B. Rosenthal, M. Alex, and E. W. Dempsey, *Anat. Rec.* **114,** 555 (1952).
[10] R. Ross and P. Bornstein, *J. Cell Biol.* **40,** 366 (1969).
[11] E. G. Cleary and M. A. Gibson, *Int. Rev. Connect. Tissue Res.* **10,** 97 (1984).
[12] B. C. Starcher and M. J. Galione, *Anal. Biochem.* **74,** 441 (1976).

have included extraction with detergent solutions, digestion with other proteolytic enzymes such as trypsin, against which elastin is resistant, and reaction with cyanogen bromide in formic acid, since it is generally agreed that most elastins contain no methionine (for exceptions see Table I). Depending on the starting tissue, one or the other of these modified procedures should be suitable for preparing acceptable elastin in the absence of alkali treatment.

Phylogenetic Distribution and Comparative Amino Acid Compositions of Elastin

A large proportion of the early work on the analysis and characterization of elastin was carried out on samples isolated from ligamentum nuchae or the aortas of mammals (see Ref. 6). It was readily apparent from these analyses that, consistent with its unique physical properties, elastin is peculiar in its chemical composition. Approximately 33% of the amino acids are glycine, 10–13% are proline, over 40% are other amino acids with hydrophobic side chains, and there are very small amounts of hydrophilic amino acids. The amino acid composition is sufficiently similar to that of collagen to suggest that it might be an unusual form of collagen. While structural work has clearly shown that this is not the case, there is some evidence for a distant homologous relationship at the amino acid sequence level (see Ref. 13 and below). Elastin preparations from different tissues of the same species have very similar amino acid compositions with the exception of that from elastic cartilage. Based on this difference, it has been suggested that the elastin in elastic cartilage may be composed of a genetically distinct polypeptide chain.[14] However, because of the particular difficulty in removing contaminating protein in cartilage, further work is necessary to substantiate this point. Foster *et al.*[15] found that the tropoelastins, the soluble biosynthetic intermediate (see below), isolated from aortic and ear cartilage of lathyritic piglets have very similar amino acid compositions.

A very extensive survey of the occurrence and amino acid composition of elastin throughout the animal kingdom was carried out by Sage and Gray,[16] who performed analyses on representative species from all vertebrate phyla and a number of invertebrate phyla. Although they prepared

[13] D. W. Smith, L. B. Sandberg, B. H. Leslie, T. B. Wolt, S. T. Minton, B. Myers, and R. B. Rucker, *Biochem. Biophys. Res. Commun.* **103,** 880 (1981).
[14] D. A. Keith, M. A. Paz, P. M. Gallop, and M. J. Glimcher, *J. Histochem. Cytochem.* **25,** 1154 (1977).
[15] J. A. Foster, C. B. Rich, and M. D. Desa, *Biochem. Biophys. Acta* **626,** 383 (1980).
[16] H. Sage and W. R. Gray, *Comp. Biochem. Physiol.* **64B,** 313 (1979).

and analyzed several tissues in a number of species, most of the assays were carried out on samples of aorta and related vessels. They utilized a relatively nondegradative procedure involving defatting of the tissue, extraction with a concentrated guanidine solution containing 2-mercaptoethanol, and finally autoclaving to purify the elastin. Where they could be compared, the resulting preparations had amino acid compositions very similar to that prepared using alkali extraction, cleavage with cyanogen bromide, or digestion with proteolytic enzymes. Their results are very clean-cut with respect to the distribution within the vertebrate phyla. Elastin is found in every vertebrate species examined, except for those in the *Agnatha* (jawless fish or cyclostome). Although the amino acid compositions of all the elastins have similar general characteristics in that they contain desmosines (cross-links characteristic of elastin), are rich in glycine, proline, and hydrophobic amino acids and poor in hydrophilic ones, there is significant variation between species within a phylum and considerable variation between phyla. Representative compositions are given in Table I, which also contains some analyses of Starcher and Galione[12] who used a different isolation method. In contrast to the previous results with mammalian and bird elastin, in which histidine, methionine, and cysteine were found to be absent, these amino acids are found in the elastin of many reptiles, amphibians, and fish. Consideration of the changes in composition during evolution suggested that the earliest elastin, which arose at some time after the divergence of the cyclostome and gnathosome lines, was similar in amino acid composition and cross-linking to that of mammalian elastin, although there has been a progressive increase in hydrophobicity with ecologic time. This trend in hydrophobic residues may be related to a parallel change in systolic blood pressure, which also increases from a low of 30 mn of mercury in fish and amphibians to 120–150 mm in mammals and birds. Hydrophobic effects may play a major role in the mechanism of elastin fiber formation and rubber-like elasticity.

In no case did Sage and Gray find elastin in invertebrate species either by chemical or histologic methods, and these findings suggest that the earlier histologic identifications of elastin based on characteristic staining reactions are probably in error. It is true that other rubber-like proteins such as resilin,[17] abductin,[18] and an octopus elastomer[19] occur in the invertebrates but they are not related to elastin, since they do not contain characteristic desmosine cross-links and have distinctly different amino acid compositions.

[17] S. O. Andersen, *Comp. Biochem.* **26C,** 633 (1971).
[18] R. E. Kelly and R. V. Rice, *Science* **155,** 208 (1967).
[19] R. E. Shadwick and J. M. Gosline, *Science* **213,** 759 (1981).

TABLE I
AMINO ACID COMPOSITION OF ELASTIN FROM AORTAS OF VARIOUS SPECIES[a,b]

Amino acid	Pig	Dog	Human[c]	Chicken[c]	Turtle	Yellow fin tuna	African lungfish
Lys	5.2	5.2	4	2	6.8	15	12
His	1.0	1.9	0.5	0.7	3.6	2.6	5.3
Arg	7.9	9.1	9	5	7.6	16	15
Asx	6.4	7.6	6	7	3.4	26	14
Thr	15	24	12	9	18	63	33
Ser	12	16	8	6	11	34	23
Glx	19	22	18	13	24	43	35
Hyp	8.7	11	10	26	16	5.5	12
Pro	113	107	131	131	130	101	112
Gly	313	314	295	338	319	391	351
Ala	244	249	233	179	184	103	105
Val	128	99	143	173	151	64	110
Ile	18	28	23	20	17	19	43
Leu	54	46	58	56	58	45	80
Tyr	19	29	23	13	34	37	27
Phe	33	25	22	19	13	28	18
Cys	<1	2.3			<0.9	<1	<0.8
Met	<1	2.1			2.0	7.1	3.5
Ide	1.9	1.8	2.2	1.2	1.5	0.3	0.7
Des	1.3	1.3	2.8	1.4	1.2	0.3	0.5

[a] Taken from Sage and Gray[16] except where indicated.
[b] Values are expressed as residues/1000.
[c] Taken from Starcher and Galione.[12]

Primary Structure

Because of cross-linking and consequent insolubility, chemical work on the structure of elastin had been greatly hampered and progressed slowly. Although elastin can be partially solubilized by nonspecific hydrolysis with weak acids or alkali, the resulting mixture of peptides (called α-elastin when oxalic acid is used) is very heterogeneous and difficult to

resolve because of the similarity in amino acid composition and chemical properties of the peptides. Surprisingly, as far back as the early 1900s, amino acid and sequence analyses were performed by Emil Fisher and coworkers and the dipeptides Ala-Leu and Leu-Ala were identified in bovine ligamentum nuchae elastin.[20] Much later in the 1960s, other dipeptides, Val-Val, Leu-Val, and Val-Pro, were identified but no extensive sequences were determined. The major significant achievements at this time were the elucidation of the structure of the desmosine cross-links by Thomas *et al.*[21] and the demonstration that these were derived from lysine residues,[22,23] as described below. The availability of highly purified preparations of pancreatic elastase and other proteases enabled elastin to be digested in a way that resulted in a complex, but somewhat less heterogeneous, collection of peptides. Anwar[24] purified peptides containing desmosines from such a peptide mixture and used preparative Edman degradation to release peptides which were C-terminal to these cross-links. Fractionation of the released peptides by ion-exchange chromatography and limited sequence determination by analytical Edman degradation yielded sequences of which the following are representative:

Porcine aorta	Bovine aorta	Human aorta
Tyr-Gly-Ala-Pro-Gly-Ala-Gly	Phe-Gly-Ala-Ala	Tyr-Gly-Ala-Ala
Ala-Pro-Gly-Gly-Gly-Gly-Ala	Ala-Gly-Tyr-Pro-Thr	Ala-Gly-Tyr-Pro-Thr
Leu-Gly-Ala-Ala	Leu-Gly-Ala-Gly-Gly-Ala	Phe-Gly-Ala-Gly

Thus, tyrosine is found frequently on the carboxyl side of cross-links (often phenylalanine in bovine elastin). Other sequences often begin with alanine and sometimes with leucine or isoleucine. Two peptides containing desmosine were isolated from a subtilisin digest of ligamentum nuchae α-elastin by Foster *et al.*[25] Sequence analysis of these peptides has suggested that each desmosine cross-links two chains, but because the two chains in the cross-linked peptides were sequenced simultaneously, the deduced sequences must be viewed as provisional. However, these analyses, as well as others, pointed out the clustering of alanine residues surrounding the lysine residues which were to form the cross-links.

[20] E. Fisher and E. Abderhalden, *Ber. Dtsch. Chem. Ges.* **40,** 3544 (1907).
[21] J. Thomas, D. F. Elsden, and S. M. Partridge, *Nature (London)* **200,** 651 (1963).
[22] E. J. Miller, G. R. Martin, and K. A. Piez, *Biochem. Biophys. Res. Commun.* **17,** 248 (1964).
[23] S. M. Partridge, D. F. Elsden, and J. Thomas, *Biochem. J.* **93,** 30c (1964).
[24] R. A. Anwar, *in* "Elastin and Elastic Tissue" (L. B. Sandberg, W. R. Gray, and C. Franzblau, eds.), p. 329. Plenum, New York 1977.
[25] J. A. Foster, L. Rubin, H. M. Kagan, E. Bruenger, and L. B. Sandberg, *J. Biol. Chem.* **249,** 6191 (1974).

Isolation of Tropoelastin

A major advance in understanding elastin chemistry came about with the isolation from the tissues of copper-deficient animals of a soluble protein that is clearly related to elastin. It had been observed in nutritional studies involving trace metals that animals on a copper-deficient diet suffered aneurysms of the aorta and other defects which could be attributed to a decreased content of the amorphous component in their elastic fibers. This led to the isolation by Smith *et al.*[26] of a soluble polypeptide from the aorta of copper-deficient pigs. The relationship of this protein, which has come to be called tropoelastin, to insoluble elastin was solidified by work of Sandberg *et al.*,[27] who showed that the amino acid composition is very similar to that of insoluble elastin except for the absence of cross-links and a corresponding increase in lysine residues. The total lysine content is 38 residues/mol in tropoelastin compared to about 6 in mature elastin. Peptide maps obtained by pancreatic elastase digestion demonstrated that although insoluble elastin and the soluble protein, tropoelastin, share a number of peptides, there are peptides which are unique to each. The differences are due to the retention in tropoelastin of many lysine residues. Tropoelastin has been isolated from copper-deficient chicks and calves and from lathyritic animals.[28–31] (Lathyrism is induced by feeding animals β-aminopropionitrile, an inhibitor of peptide lysine oxidase, see below.) The tropoelastins from all species share a number of features in addition to their similarity in amino acid composition, including a molecular weight of 72,000 to 74,000, unusually high solubility in concentrated solutions of short-chain alcohols, and a negative temperature coefficient of solubility in salt solutions.[29,31–33] The last property leads to the phenomenon of coacervation, or phase separation, of tropoelastin from a cold solution when the temperature is raised to greater than 25°.

[26] D. W. Smith, N. Weissman, and W. H. Carnes, *Biochem. Biophys. Res. Commun.* **31,** 309 (1968).
[27] L. B. Sandberg, N. Weissman, and D. W. Smith, *Biochemistry* **8,** 2940 (1969).
[28] J. A. Foster, R. Shapiro, R. C. Voynow, G. Rombie, B. Faris, and C. Franzblau, *Biochemistry* **14,** 5343 (1975).
[29] R. B. Rucker, W. Gottlich-Rieman, and K. Tom, *Biochem. Biophys. Acta* **317,** 193 (1973).
[30] B. C. Sykes and S. M. Partridge, *Biochem. J.* **141,** 567 (1974).
[31] A. H. Whiting, B. C. Sykes, and S. M. Partridge, *Biochem. J.* **141,** 573 (1974).
[32] L. B. Sandberg, R. D. Zeikus, and I. M. Coltrain, *Biochem. Biophys. Acta* **236,** 542 (1971).
[33] D. W. Smith, D. M. Brown, and W. H. Carnes, *J. Biochem.* **247,** 2427 (1972).

Porcine tropoelastin has been digested with trypsin, the peptides fractionated by ion-exchange chromatography and a number of these purified peptides sequenced by automated Edman degradation.[32,34,35] Representative sequences are illustrated in Table II. These analyses have corroborated and extended the limited sequence data on peptides from insoluble elastin. Some general conclusions can be drawn from these data as well as from other results in which tropoelastin was succinylated prior to tryptic digestion, which confines the cleavage to arginine residues from the intervening regions. Two classes of tryptic peptides were found: small ones rich in alanine are derived from regions which will form the cross-links and other larger peptides rich in hydrophobic residues are derived from the regions responsible for the elastic behavior (Table II). Two of the small peptides, Ala-Ala-Ala-Lys and Ala-Ala-Lys, are repeated six times per mol of tropoelastin while several others are repeated twice. Undoubtedly these small peptides are spaced throughout the tropoelastin separated by the larger tryptic peptides.

Provisional support for this concept has come from work on the structure of the bovine elastin gene as discussed below. Within the larger peptides, smaller limited repeats may be discerned. Such repeating sequences have raised the possibility of a secondary helical structure peculiar to elastin in a portion of the molecule,[36] but considerable evidence supports the concept that elastin is largely a random coil.[37–39] The sequences of the larger porcine peptides also show that even though glycine constitutes approximately one-third of the residues in elastin, glycine is not found regularly as every third amino acid and there are numerous occurrences of glycine residues adjacent to one another, unlike the sequences found in collagen. However, chick tropoelastin contains a collagen-like stretch of 17 Gly-X-Y triplets of which most are Gly-Val-Pro.[13] Presumably, this region cannot form a collagen triple helix, since proline is usually in the wrong position. However, this finding raises the possibility that the collagens and elastin have a distant homologous relationship at the primary structure level. Furthermore, the apparent marked difference between chick and porcine tropoelastin, also seen in the amino acid composition of many elastins (Table I), suggests that exact interspecies homology is low. This may occur because there are, presumably, many ways to make a random coil, probably the major factor in the properties of elastin, and selective constraints are less than for ordered proteins. As noted, there is a preferential occurrence of prolyl residues on the amino

[34] J. A. Foster, E. Bruenger, W. R. Gray, and L. B. Sandberg, *J. Biol. Chem.* **248**, 2876 (1973).

[35] L. B. Sandberg, N. Weissman, and W. R. Gray, *Biochemistry* **10**, 52 (1971).

[36] D. W. Urry and M. M. Long, *CRC Crit. Rev. Biochem.* **4**, 1 (1976).

[37] W. W. Fleming, C. E. Sullivan, and D. A. Torchia, *Biopolymers* **19**, 597 (1980).

[38] P. J. Flory, "Principles of Polymers Chemistry." Cornell Univ. Press, Ithica, New York, 1967.

[39] D. A. Torchia and K. A. Piez, *J. Mol. Biol.* **76**, 419 (1973).

TABLE II
SAMPLE AMINO ACID SEQUENCES FOUND IN TROPOELASTIN[a]

Repeating unit	Large tryptic hydrophobic peptides[b]
Tripeptide	Y V A G V P G V G V P G V G I G G V P G V P G V P G V P G V P G V P G V P G V P G V P G V P G V P G V P G V P G V V G G V G P V G V A A A A A A A A A
Tetrapeptide	G G V P G A V P G G V P G G V F F P G A G L G G L G
Pentapeptide	Y G A A G G L V P G A P G F G P G V G V P G V G V P G V G V P G V G V P G V S V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G A V X P A A A A K
Hexapeptide	A A Q F G L F P G I G V A P G V G V A P G V X V A P G V G V X P G V G V A P X I
None	G G V G V G G I P T F G V G A G G F P F G G V G V G G V V P G V G L P G G V Y P G

Small tryptic peptides rich in alanine[c]

Sequence	mol/mol tropoelastin
A A A K	6
A A K	6
S A K	2
A P G K	2
A K	1
Y G A K	2

[a] Single letter designation for amino acids: A, Ala; F, Phe; G, Gly; I, Ilu; K, Lys; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; Y, Tyr.

[b] Amino acid sequences from large tryptic peptides of tropoelastin showing limited repeats (underlining). The sequences are porcine[34,35] except the one showing a tripeptide repeat which is chick.[13] The proline residues preceding glycine are often partially hydroxylated (about 10%).

[c] Taken from Sandberg *et al.*[35]

side of glycine with proline found only rarely on the carboxyl side. The prolines on the amino side may be partially hydroxylated in elastin. In collagen, hydroxyproline has been shown to stabilize the triple helix,[40-42] but no known function has been found for hydroxyproline in elastin.

Cross-Linking

A most important feature of the elastic fiber, which is crucial to its proper function, is the high degree of cross-linking of the individual polypeptide chains. This cross-linking is mediated by the enzyme peptidyl lysine oxidase (lysyl oxidase) which oxidizes selective lysine residues in peptide linkage to α-aminoadipic semialdehyde (trivial name, allysine). This is the same enzyme which appears to be involved in collagen cross-linking and its isolation, purification, assay, and properties are discussed in detail by Siegel.[43] The activity of peptidyl lysine oxidase was first observed using a substrate of [6-^{3}H]lysine-labeled protein from chick aorta. The substrate was prepared by incubating the aortas with β-aminopropionitrile to inhibit endogenous peptidyl lysine oxidase and consisted of a mixture containing primarily labeled elastin with some collagen. When this substrate was incubated with an extract of embryonic chick bones, tritiated water was released and allysine was formed. In these experiments, it was not possible to determine what proportion of the total allysine was being formed in each of the two possible substrates, collagen and elastin. Subsequent experiments, using more highly purified preparations of peptidyl lysine oxidase and very pure collagen or elastin, have demonstrated that the enzyme functions better on insoluble forms of the substrates. Thus, preincubation of soluble collagen to allow aggregation, or of tropoelastin to allow coacervation, results in increased oxidation by a given amount of enzyme.

The above observation is consistent with the finding that a substantial amount of peptidyl lysine oxidase is associated with insoluble fibers in the connective tissue matrix. The enzyme can be extracted using high concentrations of urea with retention of enzymatic activity upon removal of the urea by dialysis. This enzyme–substrate complex can be visualized using fluorescenttagged antibody against lysyl oxidase. Purification of the enzyme and fractionation by either ion-exchange or molecular sieve chromatography have demonstrated a number of forms of the enzyme differing in charge and having molecular weights ranging from 3×10^4 to greater than 10^6. The higher molecular weight forms may be composed of similar subunits, but the significance of this heterogeneity is not clear since each of the components exhibits similar enzymatic activity. The finding of enzyme associated with matrix fibers substantiates the idea that it is secreted and acts in the extracellular space. When fibroblasts are grown in culture under conditions in which the majority of the collagen is secreted into the medium, peptidyl lysine oxidase is found largely in the incubation medium whereas comparatively little is associated with the cell layer, again suggesting that it is secreted and acts extracellularly.

[40] R. Berg and D. J. Prockop, *Biochem. Biophys. Res. Commun.* **52**, 115 (1973).
[41] S. Jimenez, M. Harsch, and J. Rosenbloom, *Biochem. Biophys. Res. Commun.* **52**, 106 (1973).
[42] J. Rosenbloom, M. Harsch, and S. Jimenez, *Arch. Biochem. Biophys.* **158**, 478 (1973).
[43] R. C. Siegel, *Int. Rev. Connect. Tissue Res.* **8**, 73 (1980).

Cross-link formation in elastin follows the same course as in the collagens with three major exceptions. (1) Hydroxylysine is not involved; there is none in elastin. (2) Histidine is not involved; there is little or none in elastin. (3) The final products of the series of aldol and aldimine condensations, desmosine and isodesmosine, are well characterized and are absent from the collagens.

There are two difunctional cross-links: dehydrolysinonorleucine (deLNL) formed from one residue of allysine and one of lysine, and allysine aldol (AA) formed from two residues of allysine. Their structures and that of the desmosines are shown in Fig. 2. deLNL also occurs as the reduced, and thus stabilized, secondary amine, lysinonorleucine. AA, shown as the dehydrated aldol, may be hydrated *in vivo*. A trifunctional cross-link, dehydromerodesmosine (deMD) (also shown in Fig. 2), may also be present in the reduced form *in vivo*. Desmosine and isodesmosine

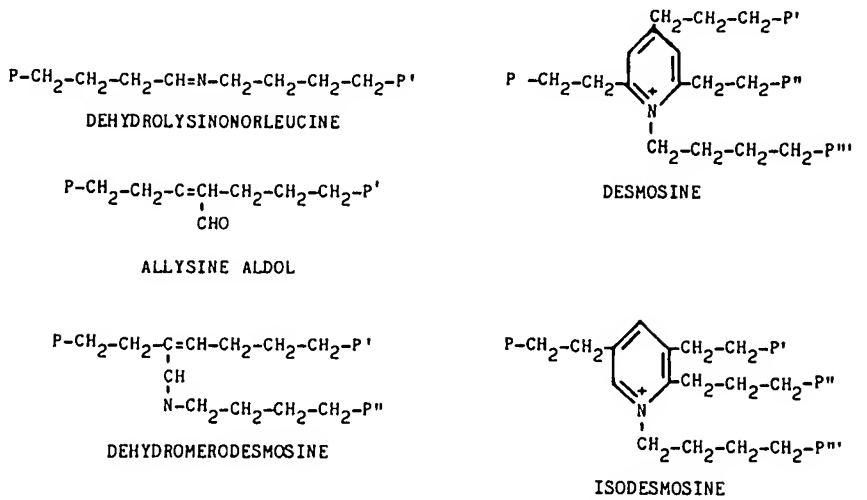

FIG. 2. Structures of elastin cross-links.

TABLE III
CROSS-LINK AND CROSS-LINK PRECURSORS IN MATURE ELASTIN

Cross-link	Lysine equivalents[a]
Desmosines	15
Merodesmosine	2
Lysinonorleucine	3
Allysine aldol	6
Allysine	2
Lysine	6
Total	34

[a] Residues/mol tropoelastin.

are tetrafunctional, but the evidence discussed earlier suggests that they normally join only two chains, two nearby lysine residues from each chain contributing to their formation. The desmosines could be formed from AA and deLNL or by several routes by the repeated addition of single residues of lysine or allysine with deMD as an intermediate. Overall, desmosine formation requires an oxidation (loss of two protons). Perhaps there is a balance in the reduction of deLNL and deMD.

Insofar as is known, all of these reactions are spontaneous. They presumably occur essentially randomly between residues in the highly mobile elastin chains. Early models in which elastin is ordered and assembles in an ordered manner remain to be proven. Table III shows an approximate balance of the various cross-links and cross-link precursors in mature elastin. About 10% of the lysine residues found in tropoelastin remains unaccounted for in elastin, either because of losses during analysis or the presence of other unidentified derivatives. The net result of the cross-linking is a highly insoluble polymer in which some type of interchain link occurs frequently along the original tropoelastin chains. Calculations from the cross-link content and from the mechanochemical properties of elastin indicate that the average distance, measured in amino acid residues, between lysine-derived cross-links is about 65–70 residues.[44]

Structure of the Elastin Gene

Although a great deal of amino acid sequences have been determined of tryptic peptides derived from porcine tropoelastin,[25,34,45] the order of those peptides has not been established. An alternative approach to elucidation of the structure of the protein is the isolation and determination of the DNA sequence of the elastin gene. Recently, sheep elastin cDNA clones containing translated sequences,[46,47] and substantial portions of the 3′ region of sheep,[48] cow,[49] and human elastin[50] genes have been obtained. Extensive DNA sequencing has demonstrated that the elastin gene appears to be one of the most dispersed genes reported so far with an intron to exon ratio of 15 : 1, which can be compared with other very dispersed genes, such as the collagen α2(I) gene with a ratio of 8 : 1.[51] Even though tropoelastin, the biosynthetic intermediate, is one-half the size of the procollagen chains, it can be estimated that the entire elastin gene will be over 40 kb, if the coding ratio is maintained. The translated exons are small, ranging from 27 up to 114 bp and, unlike the collagen genes, no regularity in exon size has been observed. The sizes of elastin exons are multiples of three and glycine is found consistently, but not invariably, at the exon–intron junctions. Hydrophobic and cross-link regions of elastin are encoded by separate exons, and these units also appear to alternate with each other, except in the region corresponding to the carboxy terminus. These findings support, in part, the hypothesis that each exon may have corresponded to a specific protein domain and that the gene arose by nonreciprocal recombination between existing exons.

There was extensive homology between the cow, sheep, and human amino acid sequences determined by DNA sequencing and the sequences derived from the tryptic peptides of porcine tropoelastin. However, the last 58 carboxy-terminal amino acids determined by DNA sequencing were not observed in the tryptic peptides. The newly determined carboxy terminus ended with the unusual sequence Gly-Gly-Ala-Cys-Leu-gly-Lys-Ser-Cys-Gly-Arg-Lys-Arg-Lys. Previous reports on the primary structure have not shown the presence of cysteine in tropoelastin. However, cysteine incorporation during *in vitro* translation of chick aorta mRNA has been reported.[52] The presence of the cysteine and of the basic

[44] B. B. Aaron and J. M. Gosline, *Biopolymers* **20**, 1247 (1981).
[45] L. B. Sandberg and J. M. Davidson, *Pept. Protein Rev.* **3**, 169 (1984).
[46] K. Yoon, M. May, N. Goldstein, Z. K. Indik, L. Oliver, C. Boyd, and J. Rosenbloom, *Biochem. Biophys. Res. Commun.* **118**, 261 (1984).
[47] K. Yoon, J. M. Davidson, C. Boyd, M. May, P. LuValle, N. Ornstein-Goldstein, J. Smith, Z. Indik, A. Ross, E. Golub, and J. Rosenbloom, *Arch. Biochem. Biophys.* **24**, 684 (1985).
[48] J. M. Davidson, S. Shibahara, M. P. Schafer, M. Harrison, C. Leach, P. Tolstoshev, and R. G. Crystal, *Biochem. J.* **220**, 643 (1984).
[49] G. Cicila, M. May, N. Ornstein-Goldstein, Z. Indik, S. Morrow, H. S. Yeh, J. Rosenbloom, and K. Yoon, *Biochemistry* **24**, 3075 (1985).
[50] N. O. Goldstein, Z. Indik, K. Yoon, G. Cicila, C. Boyd, S. Morrow, M. May, J. C. Rosenbloom, and J. Rosenbloom, *Fed. Proc.* **44**, 662 (1985).
[51] H. Boedtker, F. Fuller, and V. Tate, *Int. Rev. Connect. Tissue Res.* **10**, 1 (1983).
[52] J. A. Foster, C. B. Rich, S. Fletcher, S. R. Karr, M. D. DeSa, T. Oliver, and A. Przybyla, *Biochemistry* **20**, 3528 (1981).

residues at the carboxy-terminus of the tropoelastin suggests that this region might play an important role in the assembly of amorphous elastin and cysteine-rich, acidic microfibrillar components.

The 3′ end of the gene also contains a 973-bp-long nontranslated sequence. This region also appears to be strongly conserved between sheep, cow, and human and while we can postulate that it has some regulatory function, its function is not really known. A more detailed description of elastin gene structure is contained in this volume.[53]

Biosynthesis of Elastin

The isolation and characterization of tropoelastin from copper-deficient animals suggested that this 72,000-Da polypeptide is a soluble intermediate in the biosynthesis of insoluble elastin. However, the possibility remained that the occurrence of tropoelastin was the result of the abnormal nutritional status of the animal and that tropoelastin was an artifact, albeit a useful and informative one. In order to delineate the status of tropoelastin in the biosynthetic pathway, other types of experiments have been performed. Tissues and cells which rapidly synthesize elastin were incubated with radioactive amino acids and the soluble labeled proteins extracted and characterized by a number of techniques.[54,55] Tropoelastin was identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, solubility in aqueous alcohol solutions, labeling pattern with various amino acids including glycine, proline, alanine, and valine, resistance to cyanogen bromide degradation, and immunoprecipitation with affinity-purified antibody to insoluble elastin. It was found that approximately 20 min is required to synthesize the tropoelastin molecule and secrete it into the extracellular matrix.[56] Unlike the case of procollagen, inhibition of peptidyl proline hydroxylation does not inhibit the rate of tropoelastin secretion.[55,57] Also in contrast to procollagen, the incorporation of *cis*-hydroxyproline in place of proline does not alter secretion. However, agents such as colchicine, which depolymerize microtubules, significantly inhibit secretion.[55] Pulse-chase experiments have demonstrated that tropoelastin is incorporated into insoluble elastin and experiments designed to detect cleavage prior to incorporation have not revealed any such cleavage.[58] In all likelihood, the tropoelastin is incorporated intact onto the surface of forming fibers. Additional evidence for the legitimate role of

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[54] L. Murphy, M. Harsch, T. Mori, and J. Rosenbloom, *FEBS Lett.* **21,** 113 (1972).
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[56] J. Rosenbloom and A. Cywinski, *Biochem. Biophys. Res. Commun.* **69,** 613 (1976).
[57] J. Rosenbloom and A. Cywinski, *FEBS Lett.* **65,** 246 (1976).
[58] A. S. Narayanan and R. C. Page, *J. Biol. Chem.* **251,** 1125 (1976).

tropoelastin as a biosynthetic intermediate has come from the observation that when isolated, [^{14}C]lysine-labeled, porcine tropoelastin is incubated with fresh preparations of normal newborn pig aorta, labeled desmosines, as well as lysinonorleucine and merodesmosine, can be recovered.[59]

Although it was clear from the experiments described above that tropoelastin could be incorporated into insoluble fibers, the question remained whether it was the primary translation product, since other higher molecular weight intermediates in the 110,000–120,000 range have been reported.[60] However, when polyribosomes synthesizing elastin were isolated from embryonic chick aortas and the completion of nascent chains was carried out *in vitro*, the predominant polypeptide found had a molecular weight of 72,000.[61] Similarly, when mRNA isolated from chick embryo aortas was translated in a nuclease-treated rabbit reticulocyte lysate system and the labeled products were precipitated with elastin-specific antibody, the largest polypeptide found was slightly larger than a tropoelastin standard and no significant amounts of a higher molecular weight species were detected.[62] These experiments strongly suggest that tropoelastin is the primary translation product. The slightly larger size of the *in vitro* translation product is due to an N-terminal prepeptide or signal sequence of 24 to 26 amino acids.[63,64] Foster *et al.*[52,65] have reported that chick and pig tissues contain elastin mRNA coding for two distinct tropoelastin species, but this remains to be confirmed.

Control of elastin synthesis was investigated in the developing embryonic chick aorta and sheep nuchal ligament. In both these systems, measurements of functional elastin mRNA by translation in the nuclease-treated reticulocyte lysate demonstrated a strong correlation between messenger levels and rate of elastin synthesis in the tissue, suggesting that the rate of synthesis is controlled by the mRNA level.[65–68] Recombinant

[59] D. W. Smith, P. A. Abraham, and W. H. Carnes, *Biochem. Biophys. Res. Commun.* **66,** 893 (1975).
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[61] L. Rhyanen, P. H. Graves, G. M. Bressan, and D. J. Prockop, *Arch. Biochem. Biophys.* **185,** 344 (1978).
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[63] J. M. Davidson, B. Leslie, T. Wolt, R. G. Crystal, and L. B. Sandberg, *Arch. Biochem. Biophys.* **218,** 31 (1982).
[64] S. R. Karr and J. A. Foster, *J. Biol. Chem.* **256,** 5946 (1981).
[65] J. A. Foster, C. B. Rich, S. Fletcher, S. R. Karr, and A. Przybyla, *Biochemistry* **19,** 857 (1980).
[66] W. Burnett, R. Eichner, and J. Rosenbloom, *Biochemistry* **19,** 1106 (1980).
[67] J. M. Davidson, K. Smith, S. Shibahara, P. Tolstoshev, and R. G. Crystal, *J. Biol. Chem.* **257,** 747 (1982).
[68] J. M. Davidson, S. Shibahara, C. Boyd, M. L. Mason, P. Tolstoshev, and R. G. Crystal, *Biochem. J.* **220,** 653 (1984).

cDNA clones were obtained for chick aorta elastin mRNA and used to quantitate relative elastin mRNA concentrations in various age chick aortas. These results demonstrated that the functional mRNA levels are a reflection of the actual levels as measured by hybridization techniques and confirm the conclusion that the mRNA level controls the rate of elastin synthesis.[69] Similar results were found in the developing sheep lung and nuchal ligament.[68] In Northern hybridization experiments, cloned elastin DNA hybridizes to a 3.5-kb message.[68,69] Since tropoelastin contains approximately 830 amino acids, 2.5 kb is necessary for coding. The occurrence of an extra 1.0 kb in the message is consistent with the finding of a 973-bp untranslated 3′ region in both bovine genomic and the sheep cDNA clones discussed above. These results, considered collectively, solidify the proposition that tropoelastin is the primary elastin gene product.

The Microfibrillar Component

The above discussion has focused on elastin itself, but at least in higher vertebrate tissues with the possible exception of elastic cartilage, the elastic fiber contains microfibrils which must be presumed to have an important functional role. This microfibrillar component consists of 10- to 12-nm microfibrils which are located largely on the surface of mature fibers and to a smaller extent are enmeshed and scattered throughout the amorphous component. Definition of the molecular composition of the microfibrillar component has progressed slowly, mainly because of difficulties in solubilizing the microfibrils in any solvents other than powerful denaturants or by extraction of tissues with hot 0.1 *M* sodium hydroxide which hydrolyzes the microfibrillar protein. Milder isolation methods using sequential extraction with a concentrated guanidine solution, followed by digestion with purified collagenase and by guanidine extraction under reducing conditions, have permitted isolation of a protein fraction with an amino acid composition distinct from that of the amorphous component.[10] The soluble fraction contains large amounts of acidic amino acids as well as serine and threonine. An unusual feature of this preparation is the high content of cystine/cysteine which probably explains the marked degree of insolubility of the microfibrils in the absence of reducing agents. Incubation of the microfibrillar protein with trypsin or chymotrypsin releases soluble peptides while these enzymes do not solubilize the amorphous component to any significant extent.

[69] W. Burnett, A. Finnigan-Bunick, K. Yoon, and J. Rosenbloom, *J. Biol. Chem.* **257,** 1569 (1982).

While the extraction procedure described above differentiates the amorphous and microfibrillar components, the microfibrillar polypeptide fraction is polydisperse and no completely satisfactory characterization has been reported.[11] Another view of the chemical nature of the protein has come through use of cultured fibroblasts from bovine ligamentum nuchae. Cultured fibroblasts isolated from 45- to 135-day fetuses synthesize microfibrils resembling those in the intact tissue.[70] Isolation of protein from the medium by immunoprecipitation with antibody against a microfibrillar protein preparation revealed two proteins. One protein with a molecular weight of 140,000 to 150,000 is partly collagenous in nature, as shown by sensitivity to bacterial collagenase and incorporation of labeled proline into hydroxyproline, and may be related to type VI collagen. The apparently contradictory observations that this soluble protein is sensitive to collagenase while the microfibrillar component is resistant may be explainable by the relative resistance of type VI collagen fibrils to collagenase digestion. While these results are potentially interesting, the conclusion that these proteins are indeed related to the microfibrillar component depends critically on the monospecificity of the antibody, a technically difficult property to prove. Other workers, following similar isolation protocols, have found that the 140,000-Da component does not appear to be related to the microfibrils associated with elastin as determined by immunolocalization techniques.[71] Thus, the conclusive isolation, identification, and characterization of the elastic fiber microfibrillar component remain to be carried out.

Fiber Structure and Formation

Consistent with its chemical properties, elastin binds the usual electron microscopic stains very poorly, often not at all. Newly formed elastin, presumably because it still contains a number of unmodified lysine residues, does take up phosphotungstate, while mature elastin appears darkened only after prolonged osmification. In contrast, the microfibrillar component stains heavily with uranyl acetate and lead citrate. Under some conditions and with negative staining, amorphous elastin fibers are reported to exhibit a filamentous structure when examined at high magnification.[72,73] These filaments of 3–5 nm also may have a beaded appearance and may appear to intertwine. It is still controversial whether these fila-

[70] C. H. J. Sear, M. E. Grant, and D. S. Jackson, *Biochem. J.* **194,** 587 (1981).
[71] M. A. Gibson and E. G. Cleary, *Biochem. Biophys. Res. Commun.* **105,** 1288 (1982).
[72] G. Cleary and J. Cliff, *Exp. Mol. Pathol.* **28,** 227 (1978).
[73] L. Gotte, M. G. Giro, D. Volpin, and R. W. Horne, *J. Ultrastruct. Res.* **46,** 23 (1974).

ments represent the state of the native fiber or are artifacts caused by the drying and infiltration procedure used in negative staining.

A fundamental question arises as to the role of each of the major components in the organization and construction of the fiber. This problem has been approached by systematic studies of fetal and newborn animals in which qualitative changes have been observed in the appearance of the elastic fiber during development. These electron microscopic observations include studies on the developing rat and chick aorta, bovine ligamentum nuchae, and rat flexor tendon.[3,5,74] A general observation made in all of these studies is that the appearance of the microfibrillar component in the extracellular matrix precedes that of elastin itself. This finding has led to the hypothesis that the morphologic structure of the microfibrils determines the pattern of the mature fiber. While this is an attractive idea, the supporting evidence is purely circumstantial. Microfibrils are found consistently interspersed throughout the fiber as if they were acting as nucleating sites for the packing of soluble elastin molecules. The function of the microfibrillar component on the outside of mature fibers is not known.

The role of the cell in elastin fibrogenesis is also a matter of speculation. Based again on electron microscopic observations, newly forming elastic fibers appear to lie within folds or crevices of the plasma membrane of the synthesizing cells. This has suggested that the cell can control the orientation of the fiber by secreting elastin and/or microfibrillar protein over a limited surface of the cell in a preferred way. Earlier studies had demonstrated that cells synthesizing elastin contain numerous coated vesicles which appear to contain elastin, based on characteristic chemical staining reactions.[75] More recent experiments with chick embryo aorta using elastin-specific antibodies have confirmed and extended these studies.[76] Elastin is transported from the cisternae of the endoplasmic reticulum to the Golgi apparatus where the protein is packaged into membrane-bound vesicles. Some of these smaller vesicles appeared to fuse, forming larger vesicles which may have a storage function. Both types of vesicles were seen fusing with the cell plasma membrane, suggesting that elastin is secreted by an exocytotic process. Further details of the secretory pathway need to be determined, and it is not known whether the secretory vesicles contain elastin alone or whether other extracellular matrix components, such as the microfibrillar component or lysyl oxidase, are cosecreted within the same vesicle.

[74] T. K. Greenlee, Jr., and R. Ross, *J. Ultrastruct. Res.* **18,** 354 (1967).

[75] J. Thyberg, A. Hinek, J. Nilsson, and U. Friberg, *Histochem. J.* **11,** 1 (1979).

[76] V. Danmiano, A.-L. Tsang, G. Weinbaum, P. Christner, and J. Rosenbloom, *Collagen Rel. Res.* **4,** 153 (1984).

Degradation of Elastin

While the microfibrillar component is readily degraded by a variety of proteases, elastin is susceptible only to a few enzymes which, because of this capability, have been designated elastases. Unlike the collagenases, however, which are highly specific, the elastases are general and powerful proteases attacking a wide variety of proteins. While the first true elastases to be identified were found in bacteria and the number of organisms which have been shown to produce an elastase is considerable, their role in human disease is limited to relatively few species,[77] the most important of these being *Pseudomonas aeruginosa*.[78] The elastase produced by this organism is a metalloenzyme which preferentially cleaves at leucine and isoleucine bonds and appears to be an important factor in the pathogenesis of infection by this organism. *P. aeruginosa* produces a severe pneumonia in immunocompromised patients and in children with cystic fibrosis where it can cause severe tissue destruction. This elastase can inactivate α_1-proteinase inhibitor, thus potentiating the activity of other proteinases released by coinfecting organisms or host cells.

The first of the elastases to be identified in vertebrates and which has been characterized to a high degree was isolated from pig pancreas.[77,79] Similar enzymes are found generally in other species including man. These enzymes belong to the family of serine proteases and share their general properties, including similar reaction mechanism and inhibition by α_1-protease inhibitor and α_2-macroglobulin. Pancreatic elastase preferentially cleaves on the carboxyl side of alanine residues in elastin but also hydrolyzes peptide bonds of other hydrophobic residues. The rate of elastin degradation is markedly increased by anionic detergents such as sodium dodecyl sulfate, probably by increasing the binding of elastase to the insoluble fiber.[80] It is unlikely, under normal circumstances, that the pancreatic enzyme has a functional role other than as a digestive enzyme in the intestinal tract, since plasma α_1-protease inhibitor effectively inhibits it. When the enzyme is released in overwhelming amounts during pancreatitis, it contributes to the localized tissue destruction.

The elastase synthesized and secreted by polymorphonuclear leukocytes, first described by Janoff and Scherer,[81] readily degrades elastin,

[77] Z. Werb, M. J. Banda, J. H. McKerrow, and R. A. Sandhaus, *J. Invest. Dermatol.* **79,** 1545 (1982).

[78] N. Nishino and J. C. Powers, *J. Biol. Chem.* **255,** 3482 (1980).

[79] A. J. Barrett and J. K. McDonald, "Mammalian Proteases: A Glossary and Bibliography. Endopeptidases," Vol. 1. Academic Press, New York, 1980.

[80] H. M. Kagan, G. D. Crombie, R. E. Jordan, W. Lewis, and C. Franzblau, *Biochemistry* **11,** 3412 (1972).

[81] A. Janoff and J. Scherer, *J. Exp. Med.* **128,** 1137 (1968).

releasing peptides which differ from those released by pancreatic elastase.[82] The leukocyte enzyme is released from azurophilic granules during the inflammatory response. This enzyme is a serine protease inhibitable by α_1-proteinase inhibitor and α_2-macroglobulin. Recently, attention has been focused on an elastase prepared from macrophages which appears to be distinct from the neutrophil enzyme.[83] It is a metalloprotease inhibited by α_2-macroglobulin but not by α_1-protease inhibitor, and the cleavage products of elastin differ from those generated by either the pancreatic or leukocyte elastases. There is considerable interest in the leukocyte and macrophage enzymes since they may be intimately involved in the pathogenesis of several diseases including pulmonary emphysema.

Immunologic Studies

Elastin had been regarded for many years as a relatively poor antigen. This may in part be attributed to the techniques which were applied to test the antibodies or to the poor responses of the species which were immunized. Early studies utilized peptide antigens obtained by digestion of elastin with either alcoholic potassium hydroxide or oxalic acid.[84] In general, the resulting antisera were of low titer and contained only weakly precipitating antibodies, although they did react well in hemagglutination reactions. In later studies, antibodies were generated against insoluble chicken elastin which was administered subcutaneously to sheep as a fine suspension in complete Freund's adjuvant.[85] As observed by immunodiffusion, these antibody preparations cross-reacted with purified tropoelastin prepared from lathyritic chicks, although no precipitin line could be observed with chick α-elastin. Antibodies to insoluble human and dog elastin have also been prepared in sheep.[86] However, these antibodies were observed, by passive hemagglutination and enzyme-linked immunosorbent assay, to cross-react with α-elastin prepared from the insoluble elastin used as the immunogen. Similarly, antibodies against insoluble ligamentum nuchae reacted with α-elastin labeled with ^{125}I in a radioimmunoassay.[87] Thus, the technique of observation and the sensitivity of the

[82] R. M. Senior, D. R. Bielefeld, and B. C. Starcher, *Biochem. Biophys. Res. Commun.* **72,** 1327 (1976).
[83] M. J. Banda and F. Werb, *Biochem. J.* **193,** 589 (1981).
[84] L. Robert, J. Parlebas, N. Poullain, and B. Robert, in "Protides of the Biological Fluids" (H. Peters, ed.), p. 109. Elsevier, Amsterdam, 1963.
[85] B. Sykes and J. W. Chidlow, *FEBS Lett.* **47,** 222 (1974).
[86] U. Kucich, P. Christner, M. Lippmann, A. Fein, A. Goldberg, P. Kimbel, G. Weinbaum, and J. Rosenbloom, *Am. Rev. Respir. Dis.* **127,** S28 (1983).
[87] R. P. Mecham and B. Lange, *Connect. Tissue Res.* **7,** 247 (1980).

method may determine the ability to detect the reactivity of antibody preparations with various forms of antigen. In general, precipitating antibodies are not obtained and appropriate techniques for detecting soluble antigen–antibody complexes must be used.

Various forms of soluble elastin have been used to generate antibodies, including tropoelastin, α-elastin, and mixtures of soluble peptides obtained from purified insoluble elastin by digestion with pancreatic or neutrophil elastase.[86–88] Antisera against pig tropoelastin cross-react strongly with pig α-elastin. While the antibodies generated against peptides obtained by leukocyte elastase digestion of human elastin cross-react with those generated by pancreatic elastase and vice versa, they do not react at all with α-elastin. Antibodies to α-elastin cross-react poorly with the peptides in elastase digests. Thus, the antigenic sites in the peptides from elastase digestion are not represented in α-elastin and the antigenic sites in α-elastin are poorly represented in the elastase-derived peptides. The fact that the desmosines and other cross-links are found in all of these solubilized forms of elastin implies that at least some, and perhaps a substantial proportion, of the antibody molecules are directed against sites other than the cross-links. This conclusion is reinforced by the observation that antibodies to insoluble elastin cross-react well with tropoelastin which contains no cross-links. These considerations do not preclude formation of antibodies against antigenic sites containing desmosines or other cross-links, and in some cases these antibodies may even be the predominate species. Interestingly, attempts to elicit antibodies against the free desmosines have been unsuccessful, but when desmosine has been covalently linked to serum albumin as carrier, very high titer antisera have been obtained. These antisera to desmosine react only with free desmosine and not with desmosine in peptide linkage or with isodesmosine in any form.[89]

In general, antielastin antibodies do not show tissue specificity with respect to the source of elastin within a given vertebrate species. Thus, analysis with antibodies has not provided any evidence for the presence of tissue-specific forms of elastin, although recent evidence suggests that more than one type of elastin polypeptide chain may occur in a single species.[52,65,90] Although antibody preparations frequently appear to react only with the elastin from the species which was the source of immunogen, this may again be a reflection of the test procedures used. For exam-

[88] R. A. Daynes, M. Thomas, V. L. Alvarez, and L. B. Sandberg, *Connect. Tissue Res.* **5,** 75 (1977).
[89] G. S. King, V. S. Mohan, and B. C. Starcher, *Connect. Tissue Res.* **7,** 263 (1980).
[90] L. L. Barrineau, C. B. Rich, A. Przybyla, and J. A. Foster, *Dev. Biol.* **87,** 46 (1981).

ple, antibody against bovine ligamentum nuchae appeared to react only with forms of bovine α-elastin as the test antigen when the α-elastins were unlabeled. However, when labeled α-elastin from several other mammalian species was tested, cross-reaction was easily demonstrated. In this particular case, two classes of antibody molecules appeared to be present: a class which was bovine specific corresponding to that of the immunogen source, and a second class which reacted with antigenic sites shared by several species.[87] Fractionation of peptides prepared by thermolysin digestion of ligamentum nuchae demonstrated that the species-common antigenic determinants were associated with cross-linking regions while species-specific determinants were associated with hydrophobic regions.[91]

The development of antielastin antibodies has provided a useful tool in the investigation of the biosynthesis, localization, and degradation of elastin. Thus, antibodies have been used to identify labeled and unlabeled tropoelastin synthesized by organ and cell cultures and to immunoprecipitate polypeptides synthesized by *in vitro* systems in response to mRNA addition.[61,67,69] Because of the lack of a characteristic ultrastructure in the elastic fiber, identification and localization of elastin cannot always be readily made. Specific double antibody techniques have been developed for identification of elastin both intracellularly and in the extracellular matrix at the light and ultrastructural levels.[76,92] Since many of the antibody preparations react with degradation products of insoluble elastin, they hold the promise of being useful in problems where biological degradation may be an important feature.[86]

Summary and Perspectives

The elastic properties of many tissues such as the lung, dermis, and large blood vessels are due to the presence of elastic fibers in the extracellular space. These fibers have been shown by biochemical and ultrastructural analysis to be comprised of two distinct components, a more abundant amorphous component and the microfibrillar component. The microfibrillar component is found in 10- to 20-nm fibrils which are located primarily around the periphery of the amorphous component but, to some extent, interspersed within it. The protein, elastin, makes up the highly

[91] R. P. Mecham and G. Lange, *Biochemistry* **21**, 669 (1982).
[92] V. V. Damiano, A. Tsang, P. Christner, J. Rosenbloom, and G. Weinbaum, *Am. J. Pathol.* **96**, 439 (1979).

insoluble amorphous component and is responsible for the elastic properties. Elastin is found throughout the vertebrate kingdom except for very primitive fish and possesses an unusual chemical composition consonant with its characteristic physical properties. Elastin is composed largely of glycine, proline, and other hydrophobic residues and contains multiple lysine-derived cross-links, such as the desmosines, which link the individual polypeptide chains into a rubber-like network. The intervening, hydrophobic regions of the polypeptide chains between the cross-links are highly mobile, and the elastic properties of the fibers can be described in terms of the theory of rubber elasticity.

Recent application of recombinant DNA techniques has led to further understanding of the structure of elastin. Analyses of the bovine and human elastin genes have demonstrated that the hydrophobic and cross-linking domains are encoded in separate exons. These exons tend to be small, varying from 27 to 114 bp, and are separated by large intervening sequences. Furthermore, DNA sequence analysis has demonstrated that the elastin molecule contains two cysteine residues which were not previously identified near the carboxy-terminus and which may be important in the interaction of elastin with other extracellular matrix proteins. Further DNA sequencing should determine the complete amino acid sequence of elastin. Biosynthetic studies and *in vitro* translation of elastin mRNA have demonstrated that a 72,000-Da polypeptide designated tropoelastin is the initial translation product. Analysis of several developing systems has demonstrated that elastin synthesis is controlled by the level of elastin mRNA. After packaging into membrane-bound vesicles in the Golgi apparatus, tropoelastin is secreted by exocytosis into the extracellular space where it is cross-linked by a copper-requiring extracellular enzyme, lysyl oxidase.

Elastin can be solubilized only by proteases which have consequently been designated elastases, although these are general, powerful proteases which can hydrolyze numerous proteins. The elastase secreted by leukocytes is a serine protease inhibitable by α_1-protease inhibitor while the elastase secreted by macrophages is a metalloprotease not inhibitable by α_1-protease inhibitor. Degradation of elastin by these elastases may be central to the pathogenesis of chronic obstructive lung disease and some vascular diseases. Although no heritable diseases have as yet been shown to be caused by molecular defects of elastin, several diseases, including the Marfan syndrome, pseudoxanthoma elasticum, and the Buschke–Ollendorf syndrome, may result from such defects. Studies utilizing cloned genomic, human elastin DNA may lead to definition of genetic alterations. Similar studies of polymorphism of the elastin gene in the normal popula-

tion may identify variants which increase the susceptibility to acquired diseases which affect elastic connective tissue.

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[10] Isolation and Characterization of Insoluble and Soluble Elastins[1]

By NORMAN T. SOSKEL, TERRIL B. WOLT, and LAWRENCE B. SANDBERG

"Comprehension of function is dependent on knowledge of structure and composition" (Richards and Gies).[1a]

Extraction of Insoluble Elastin

Definition of Elastin

Elastin demonstrates remarkable functionality, correlating with macroscopic and microscopic structure and with molecular composition. Its ubiquitousness necessitates our understanding of these relationships, because tissue-specific extraction methods are necessary for its purification. Lack of agreement on criteria of purity stems from its complex supramolecular organization and intricate relationships with other macromolecules primarily of connective tissues. This further complicates its definition. In contrast to a single gene product as appears to exist for its precursor tropoelastin, insoluble elastin represents a postranslationally modified, heavily cross-linked meshwork, perhaps best described as a fiber rather than a protein. It is an infinitely large polymer. Uniform amino acid composition exists in this network with most preparations, but due to the intimate association with nontropoelastin gene products, amino acid analyses may vary with the various tissues in which it is found. Extraction methods, tailored to specific tissues, partially normalize these differences, but also introduce variable degrees of degradation into the network resulting in more varied and artifactual compositions. This raises questions concerning the validity of comparisons of elastin among various types of tissues and among various species. These questions are currently unresolved, but will be answered when the gene for the precursor, tropoelastin, has been sequenced and translated in each tissue and species in question. Thus, the current criteria of purity are not new and depend upon the unique insolubility of elastin in various solvents and its resistance to harsh degradative procedures as well as various proteolytic enzymes in comparison to other substances present in its tissue matrix.

Purity is assessed by absence of collagen and collagen-like features (methionine, hydroxylysine, high hydroxyproline), absence of carbohydrate, presence of high content of nonpolar amino acids (glycine, alanine, proline, valine), and low content of polar amino acids (aspartate, glutamate, lysine, histidine, arginine). While these are relative criteria, comparisons are often made to published analyses of tropoelastin and ligamentum nuchae or aortic-insoluble elastin (Table I).

[1] This chapter is dedicated to Dr. S. M. Partridge who was the first investigator to scientifically approach the problems associated with elastin research and who truly opened up this area of investigation.

[1a] A. N. Richards and W. J. Gies, *Am. J. Physiol.* **7**, 93 (1902).

Perspective

Richards and Gies[1] claim that Tilanus, using boiling acetic acid to remove collagen, was the first to extract elastin and that W. Muller added boiling in dilute alkali and cold mineral acid treatment, but the method of Lowrey[2] and Lansing's modification thereof[3] are the earliest methods remaining in use today. Partridge, who developed a milder method of extraction, launched a series of studies[4–6] using rigorous scientific methods to ascertain many of the structural and biochemical features we currently ascribe to elastin. By so doing, he opened up the area of elastin research and provided early predictions about the putative precursor and its posttranslational modifications.

Major Methods in Current Usage

Surprisingly few unique methods have been developed to remove tissues from elastin. Variations occur in degrees of harshness, and the choice of methods depends upon the tissue and species utilized and upon the use one plans for the residue. Below are described the major methods in current usage and their applications.

As Jackson and Cleary[7] point out, all extractions are more effective if

[2] O. H. Lowrey, D. R. Gilligan, and E. M. Katersky, *J. Biol. Chem.* **139**, 795 (1941).
[3] A. I. Lansing, T. B. Rosenthal, M. Alex, and E. W. Dempsey, *Anat. Rec.* **114**, 555 (1952).
[4] S. M. Partridge, H. F. Davis, and G. S. Adair, *Biochem. J.* **61**, 11 (1955).
[5] S. M. Partridge and H. F. Davis, *Biochem. J.* **61**, 21 (1955).
[6] G. S. Adair, H. F. Davis, and S. M. Partridge, *Nature (London)* **167**, 605 (1961).
[7] D. S. Jackson and E. G. Cleary, *Methods Biochem. Anal.* **15**, 25 (1967).

tion may identify variants which increase the susceptibility to acquired diseases which affect elastic connective tissue.

Acknowledgments

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[10] Isolation and Characterization of Insoluble and Soluble Elastins[1]

By NORMAN T. SOSKEL, TERRIL B. WOLT, and LAWRENCE B. SANDBERG

"Comprehension of function is dependent on knowledge of structure and composition" (Richards and Gies).[1a]

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[6] G. S. Adair, H. F. Davis, and S. M. Partridge, *Nature (London)* **167**, 605 (1961).

[7] D. S. Jackson and E. G. Cleary, *Methods Biochem. Anal.* **15**, 25 (1967).

TABLE I
AMINO ACID COMPOSITIONS IN RESIDUES/1000 RESIDUES FOR PREPARATIONS OF TROPOELASTIN AND INSOLUBLE ELASTIN

Amino acid	Pig TE[a] aortic[b]	Chick TE aortic[c]	Bovine E[d] ligament[e]	Pig E aortic[f]	Hamster E lung[g]
Hyp	9.3	8	9	11.0	14.3
Asp	3.5	5	7	6.2	4.2
Thr	13.1	10	9	13.6	13.5
Ser	9.6	8	9	11.4	13.0
Glu	14.5	13	17	19.1	18.7
Pro	113.9	125	116	116.9	111.0
Gly	306.2	329	314	329.9	372.7
Ala	233.1	172	242	234.1	213.6
Cys	—	—	—	—	—
Val	129.2	174	141	119.7	96.7
Met	—	—	—	0.8	2.6
Ile	17.3	18	26	17.8	31.1
Leu	51.0	53	54	54.2	53.2
Tyr	16.8	11	8	15.9	19.4
Phe	30.4	29	30	33.0	17.7
Lys	46.6	41	8	6.2	5.1
His	—	—	1	0.5	2.4
Arg	4.8	6	6	6.1	8.6
Ides[h]	—	—	1.1	1.2	0.8
Des[h]	—	—	1.4	1.8	1.2
Lnl[i]	—	—	0.5	0.9	—

[a] From Sandberg and Wolt.[8]
[b] TE represents tropoelastin.
[c] Modified from Rucker.[37]
[d] E represents insoluble elastin.
[e] Modified from Paz *et al.*[22]
[f] From Rasmussen *et al.*[14]
[g] Modified from Soskel and Sandberg.[9]
[h] Elastin cross-links isodesmosine and desmosine are expressed as leucine equivalents divided by 3.65, or as lysine equivalents divided by 4. It is suggested that either of these methods be used for calculation. The reader is warned to be careful regarding interpretation of cross-link data for considerable ambiguity and differences are seen in the literature as to how these are expressed.
[i] The elastin cross-link lysinonorleucine is expressed as leucine equivalents divided by 2.

tissues are finely divided initially. Tissue, fresh or recently thawed, is dissected free of adipose and other extraneous substances and washed with 0.15 *M* NaCl or phosphate-buffered saline. Tissue is then minced and ground to a powder in liquid nitrogen using a mortar and pestle or other mechanical pulverization device.[8]

[8] L. B. Sandberg and T. B. Wolt, this series, Vol. 82, p. 657.

Hot Alkali (Lansing's Modification of Lowrey's Method[2,3])

Materials

Ethanol
Diethyl ether (alternately chloroform and methanol)
0.1 *N* NaOH
Boiling water bath

Method. Starting material may be fresh tissue or defatted tissue. Removal of fat can be accomplished by refluxing in ether or by sequential extractions in 100% ethanol × 2, 50:50 (v/v) ether:ethanol × 2, then 100% ether × 2. Alternately, ethanol, then 2:1 (v/v) chloroform:methanol can be used. Partridge[4] differs from Jackson and Cleary[7] by stating that prior history of the starting material is important.

Wet tissue or a weighed aliquot of dry defatted tissue is minced in a small volume of 0.1 *N* NaOH at 4° and 9 volumes of 0.1 *N* NaOH is added to this. Various scales of extraction can be used but we routinely use 15- or 60-ml polypropylene culture tubes from Corning, filled not more than one-half to two-thirds full. Magnetic stir bars placed at the bottom of these conical tubes in a water bath provide sufficient mixing to prevent clumping. The water bath is heated to boiling, then the tubes containing samples and alkali are added. Timing begins when the bath starts boiling again. Tubes are removed to cool at room temperature after exactly 45 min. Jackson and Cleary[7] boil for 50 min to ensure minimal contamination while minimizing elastin degradation which begins to occur after 60 min.[3] Samples are washed with cold 0.1 *N* NaOH × 2 in a Büchner funnel or by centrifugation and then with cold double distilled water (DDW). They are then dried over P_2O_5 or lyophilized and brought to constant weight for gravimetric quantitation. For amino acid analyses as an assessment of purify, small (<1 mg) aliquots of samples may be hydrolyzed in 6 *N* HCl *in vacuo* under nitrogen at 110° for 72 hr. This amount of time is required to break all the peptide bonds in this hydrophobic protein; shorter periods (24 hr) minimize other hydrolytic losses, e.g., aspartate, glutamate, serine, threonine, hydroxyproline, and tyrosine, and may be more desirable. Amino acids may be converted to residue weights and summed to obtain total amount of protein as an alternative to gravimetric quantitation.

Comment. This procedure works well for ligamentum nuchae elastin, providing a consistent amino acid analysis (Table I). Monitoring with constant amino acid analysis is the best quality control available to us in the preparation of insoluble elastins. Lung elastin is fairly pure but the product is degraded considerably based on N-terminal analysis.[9] Quanti-

[9] N. T. Soskel and L. B. Sandberg, *Exp. Lung Res.* **4**, 109 (1983).

tation of total elastin using desmosine and isodesmosine absolute weights times a constant is fairly reliable for tissues such as lung.[10] Total amino acid analyses of lung and aorta are occasionally contaminated with collagen components (high hydroxyproline, hydroxylysine, and methionine). Quantitation of total tissue elastin by this method is suitable even for small amounts of tissue (<1 mg) when performed in 1.5-ml microfuge tubes. In such cases some of the washes and fat extractions may be eliminated in order to minimize losses. Amino acid analysis of hydrolyzed material may then be performed using N-terminal amino derivatization with phenyl isothiocyanate (PITC), *o*-phthaldialdehyde (OPA), dansyl chloride (DNS-HCl), ninhydrin, or others. Alternately, conventional ion-exchange amino acid analyzers can be used if the tissue is elastin rich. Using reversed phase HPLC (high-pressure liquid chromatography), as little as 0.5 μg of protein can be analyzed quantitatively. At least 5 μg is needed for modern conventional ion-exchange amino acid analyses. When using small amounts of tissue, the ability to obtain accurate wet and dry weights becomes the major limitation, although quantitation of total amino acids may obviate this problem if an aliquot can be spared for the analysis.

Autoclaving[4]

Materials

1% (w/v) NaCl
Ethanol
Diethyl ether
Autoclave
10% trichloroacetic acid
0.02 *M* Tris, pH 8.4, containing 0.14 *M* NaCl

Method. The autoclaving method popularized by Partridge is a milder method producing a product that is less degraded than that of the alkali method but often containing appreciable collagen contamination, especially if aorta is the starting material. Tissue is washed with 20 volumes of 1% NaCl until no protein is detected in the supernatants using the Lowrey, biuret, or other such similar protein assay. It is then washed several times with DDW and placed in centrifuge tubes, stoppered with a loose-fitting gauze plug, and autoclaved in 20 volumes DDW at 1 atm for 45 min. Repeat autoclaving with fresh DDW is performed until no further protein is detected in the supernatant (about 3–4×). The residue is dried with ethanol, lipid extracted with equal parts of ethanol/ether and then with pure ether, dried first in room air and then over P_2O_5, and finally

[10] J. A. Foster, C. B. Rich, S. B. Karr, and M. D. Desa, *Connect. Tissue Res.* **8**, 259 (1981).

milled to a fine powder in a Wiley mill. Partridge again autoclaved the material at 1 atm for 45 min for two further periods to remove traces of collagen and glycosaminoglycans. Again the material was washed with warm DDW, dehydrated with ethanol/ether, dried, and milled. The elastin prepared in this way is cream colored and free flowing and upon microscopic inspection is uniform in the thickness of the rod-shaped fibers. This material is suitable for solubilization with oxalic acid or alcoholic KOH[11] as is the hot alkali-extracted material, if soluble, heterogeneous elastin peptide fragments are desired.[4–6]

Comments. Jackson and Cleary[7] recall that they, as well as others, have extracted 2–8% more nonelastin material from this preparation using Lansing-type purification and that traces of collagen are frequently observed. They suggest more rigorous initial tissue preparation by extracting in 0.02 *M* Tris, pH 8.4, containing 0.14 *M* NaCl. Extractions are continued until supernates are TCA negative (a clear solution when mixing equal volumes of 10% trichloroacetic acid and the extraction liquor) after which they are extracted two more times with cold DDW. Fetal tissues often require many more (up to 16) initial extractions in order to produce TCA-negative supernatants. Furthermore, because elastin appears flocculent after autoclaving, decanting may be replaced with suction using a Pasteur pipet. Occasionally, centrifugation at 100,000 *g* for 1 hr may be necessary.

Method of Ross and Bornstein[12]

Materials

5 *M* guanidine hydrochloride, pH 7
0.1 *N* NaOH
0.2 *M* Tris, pH 7.4
Pure clostridial collagenase
Penicillin/streptomycin
Shaking water bath 37°
0.1 *M* Tris, pH 8.5, containing 0.1% (w/v) ethylenediaminetetraacetic acid and 0.05 *M* dithioerythritol

Methods. This method was introduced primarily to obtain a preparation containing elastin-associated microfibrils. However, the initial preparation yields a product used by many as insoluble elastin. Starting tissue wet weight is 4 g. It is minced and homogenized in 5 *M* guanidine hydrochloride (bringing pH to 7.0 with 0.1 *N* NaOH), adding more 5 *M* guanidine hydrochloride to bring the total volume to 80 ml. It is stirred for 24 hr

[11] R. P. Mecham and G. Lange, this series, Vol. 82, p. 744.
[12] R. Ross and P. Bornstein, *J. Cell Biol.* **40**, 366 (1969).

at room temperature and then centrifuged at 2000 rpm. It is resuspended in the same volume of 5 *M* guanidine hydrochloride and the extraction repeated 2 times for a total of 72 hr extraction. The final pellet is washed with 0.2 *M* Tris (pH 7.4) × 3 and three times with DDW. Collagenase is added to 1% of the tissue dry starting weight, the pellet being suspended in 0.2 *M* Tris, pH 7.4, with 0.5% penicillin–streptomycin. After incubating at 37° for 24 hr, the mixture is centrifuged at 35,000 *g* for 20 min at 15°, dialyzed at 4° against water, centrifuged, and the pellet lyophilized.

Comment. Cleary has pointed out recently that this material still contains ultrastructurally observable elastin-associated microfibrils.[13]

Method of Rasmussen[14]

Materials

5 to 5.2 *M* guanidine hydrochloride, pH 7.2–7.3 containing 1% (v/v) 2-mercaptoethanol
Wrist-action shaker
Autoclave
97% formic acid
Cyanogen bromide

Method. The powdered tissue is extracted in 5–5.2 *M* guanidine hydrochloride, pH 7.2–7.3, containing 1% 2-mercaptoethanol at 25° for 24 hr on a wrist-action shaker. Five grams powdered tissue is used/50 ml solvent. A good grade of guanidine hydrochloride can be purchased from Calbiochem and other companies since the original report so that purification of the reagent is no longer necessary. The suspended tissue is diluted to 200 ml after 24 hr with DDW and centrifuged at 870,000 *g*-min. The pellet is suspended in 100 ml DDW and autoclaved for 5 hr at 20 psi (120°). It is filtered while hot and washed with boiling DDW using 100 ml × 3. It is then dried over P_2O_5, suspended in 50 ml 97% formic acid with excess cyanogen bromide (about 500 mg), and placed again on the wrist-action shaker at room temperature for 24 hr. At the end of that period it is diluted with 150 ml DDW, filtered, washed to neutrality with DDW, and dried.

Comment. Guanidine hydrochloride removes glycoproteins and soluble collagen. The reducing agent solubilizes the microfibrillar component. Cyanogen bromide–formate removes 1 to 10% of the total wet weight and takes advantage of the lack of methionine in elastin. All other methionine-containing proteins (collagen included) will be solubilized as they are cleaved into peptides at this residue. This method is milder than other methods, yet is rigorous enough to produce a relatively pure product from ligamentum nuchae and aorta. Lung material is still not as pure as that

[13] E. G. Cleary and M. A. Gibson, *Int. Rev. Connect. Tissue Res.* **10**, 97 (1983).
[14] B. L. Rasmussen, E. Bruenger, and L. B. Sandberg, *Anal. Biochem.* **64**, 255 (1975).

obtained using the Starcher method (see below). N-terminal analysis gives results intermediate between the other methods for lung.[9]

Method of Starcher[15]

Materials

0.05 *M* Na_2HPO_4 pH 7.6, containing 1% (w/v) NaCl and 0.1% (w/v) ethylenediaminetetraacetic acid
Autoclave
0.1 *M* Tris, pH 8.2, containing 0.02 *M* $CaCl_2$
Trypsin (Sigma, 2× crystallized)
97% formic acid
Cyanogen bromide
0.05 *M* Tris, pH 8, containing 6 *M* urea and 0.5% (v/v) 2-mercaptoethanol
Ethanol
Acetone

Procedure. Powdered tissue is extracted for 72 hr at 4° with multiple changes of 0.5 *M* Na_2HPO_4, pH 7.6, containing 1% NaCl and 0.1% EDTA. After centrifugation the pellet is washed with DDW × 2 and lyophilized. Into 30 ml of DDW, 200 mg of the lyophilisate is autoclaved for 45 min at 25 psi. After centrifugation, the supernate is discarded and the pellet washed × 2 with DDW and then placed in 30 ml 0.1 *M* Tris, pH 8.2, containing 0.02 *M* $CaCl_2$. Proteolysis is accomplished by adding 4 mg trypsin and incubating at 37° for 18 hr, after which the sample is again centrifuged, washed × 2 with DDW, and placed in 10 ml 97% formic acid containing 200 mg cyanogen bromide. After shaking at room temperature for 5 hr, the sample is centrifuged, washed × 2 with DDW, placed in 20 ml 0.05 *M* Tris, pH 8, with 6 *M* urea and 0.5% (v/v) 2-mercaptoethanol, and stirred overnight at room temperature. It is finally centrifuged, washed exhaustively with DDW, then with ethanol, then acetone, and dried *in vacuo* over P_2O_5.

Comment. Trypsin is necessary for purification of elastin from lung and uterus. SDS was not used because of the difficulty in removing it from the purified protein. N-terminal analysis was entirely negative in Starcher's paper and we too found minimal amounts compared with other methods of extraction using lung tissue.[9] This method is reminiscent of that of John and Thomas[16] with the substitution of autoclaving in place of collagenase treatment to remove collagen. The use of formic acid is derived from the methods of Hass,[17] Ayer,[18] and Rasmussen[14] (see above).

[15] B. C. Starcher and M. J. Galione, *Anal. Biochem.* **74**, 441 (1976).
[16] R. John and J. Thomas, *Biochem. J.* **127**, 261 (1972).
[17] G. M. Hass, *Arch. Path.* **34**, 807 (1942).
[18] J. P. Ayer, *AMA Arch. Pathol.* **65**, 519 (1958).

Other Methods and Special Applications

Methods of Paz,[19] *Richmond,*[20] *and Serafini-Fracasini et al.*[21] Paz[19] and Richmond[20] have developed methods specifically designed for aortic and lung extractions. These methods are derived from that of Ross and Bornstein[12] with a few additions. The products are relatively intact but are not free of collagen contamination. Serafini-Fracasini *et al.*[21] have developed another variation using a very pure collagenase which yields as pure a product as is possible to obtain, although the methodology is tedious and cumbersome to perform.

Ear Cartilage. Ear cartilage presents a particularly difficult problem due to the abundance of proteoglycans present in that tissue. A recent method deals with this problem,[22] and removal of elastin-associated microfibrils has been attempted using modifications of the guanidine extractions.[13] These methods are new and require further confirmation before general recommendation of usage.

Assessment of Results of Various Major Methods (see Table II)

Quantitative. As implied in the descriptions above, gravimetric determinations of the amount of elastin present in a tissue is satisfactory if sufficient material is available to yield a precise measurement (at least 0.1 mg dry weight). The limiting factor becomes the ability to weigh wet or dry tissue when using small samples. Quantitating the total amino acids present in a hydrolyzate of an aliquot or the whole sample is satisfactory and is in fact necessary for smaller samples. Newer methods of N-terminal derivatization and HPLC separations provide more sensitive analytical tools.[23,24] Immunologic methods such as radioimmunoprecipitation[25] and rocket immunoelectrophoresis[10] may prove to be at least as sensitive in determining the amount of material present. However, due to the insoluble nature of mature elastin, many of these methods are not useful unless elastin is present in a degraded soluble form. Cross-link analysis may also be helpful for quantitation if hydrolyzed samples can be used.[26]

Qualitative. Comparison to alkali-extracted bovine ligamentum nuchae or porcine aortic elastin by amino acid analysis is the gold standard

[19] M. A. Paz, D. A. Keith, H. P. Traverso, and P. M. Gallop, *Biochemistry* **15,** 4912 (1976).
[20] V. Richmond, *Biochim. Biophys. Acta* **351,** 173 (1974).
[21] A. Serafini-Fracasini, J. M. Field, and M. Spina, *Biochim. Biophys. Acta* **386,** 80 (1975).
[22] M. A. Paz, D. A. Keith, and P. M. Gallop, this series, Vol. 82, p. 571.
[23] V. L. Alvarez and L. B. Sandberg, *Adv. Exp. Biol. Med.* **79,** 589 (1977).
[24] M. N. Margolies and A. Brower, *J. Chromatogr.* **148,** 429 (1978).
[25] J. Rosenbloom, this series, Vol. 82, p. 716.
[26] M. A. Paz, D. A. Keith, and P. M. Gallop, this series, Vol. 82, p. 571.

TABLE II
METHODS FOR EXTRACTING INSOLUBLE ELASTIN FROM VARIOUS TISSUES

Tissue	Acceptable method	
	Quantitative studies[a]	Structural studies[b]
Ligamentum nuchae	Lansing[3]	Autoclaving[4], Rasmussen[14]
Aorta	Lansing, Rasmussen	Rasmussen
Lung	Lansing, Starcher[15]	Starcher
Skin	Lansing	?
Tendon and fascia	Lansing, Starcher	?
Ear cartilage	None[c]	None[c]
Muscle	None	None

[a] Quantitative studies require only a pure product, not necessarily an intact one, especially if quantitation is determined by desmosine analysis.
[b] Qualitative studies require a product that is both intact and free of contamination with nonelastin substances.
[c] Cleary's[13] and Paz'[22] newer procedures appear promising, but due to their newness can be recommended only with caution at this time.

by which elastin's purity is assessed (Table I). Relative paucity of basic amino acids (histidine, arginine, lysine), and high amounts of nonpolar amino acids (glycine, alanine, proline, valine, leucine, and phenylalanine), and absence of methionine are good criteria. Absence of cysteine is no longer an acceptable criterion because tropoelastin contains two of these residues based on its gene sequence.[27]

N-terminal analysis has been used successfully to determine the relative degree of degradation of extracted elastin and, hence, the harshness of the extraction procedure.[9,23] This information can now be obtained by using the derivatization methods and chromatographic separation methods of standard reversed-phase chromatography.[23,24]

Purification of Soluble Elastin

Perspective

In dealing with soluble elastin we have a protein with very unusual physical and chemical properties. The extreme hydrophobicity of large portions of the molecule results in unusual solvation and solution proper-

[27] K. Yoon, M. May, N. Goldstein, A. M. Indik, L. Oliver, and C. Boyd, *Biochem. Biophys. Res. Commun.* **118,** 261 (1984).

ties and gives a propensity toward unusual aggregation phenomena under elevated temperatures and salt conditions. These properties afford minimal difficulties in isolation and in some ways lend themselves toward the use of simple, rapid, nondegradative techniques for isolation of the protein in relatively pure form. On the other hand, the unique physicochemical properties make the molecule unusually sensitive to enzymatic degradation and thus steps *must* be taken toward preventing these undesireable complications during the isolation procedure. In fact, lack of attention to these properties can lead to completely negative results during situations in which reasonable protein yields would be expected. In addition, one or two nicks in the molecule can lead to very erroneous results when using the isolated protein as standards for electrophoresis or competitive binding assays. Thus, the techniques presented below will address the preservation of the molecular structure as a primary requisit for acceptable isolation.

Definitions

Some confusion exists in the literature regarding nomenclature applied to various soluble forms of elastin. *α-Elastin* and *κ-elastin* are nonspecific heterogeneous peptides of elastin derived by random cleavage of the elastic fiber with oxalic acid or alcoholic KOH, respectively.[10] *Tropoelastin*[28] or *salt-soluble elastin*[29] is the designation for the 70-kDa monomeric form of the protein as it occurs in tissues before cross-linking and incorporation into the insoluble elastin fiber. *Proelastin* is a term coined by some investigators to describe a putative large precursor of tropoelastin of approximately 150 kDa which is now felt by most to be an artifact of the isolation procedures (see discussion by Foster *et al.*[30]). *Elastin mRNA cell-free translation product* or *preelastin* is a 72 kDa product containing a 26 to 28 amino acid leader sequence attached directly to the N-terminal portion of tropoelastin. It is cleaved intracellularly on the rough endoplasmic reticulum and thus never leaves the internal environment of the cell.[31,32] The term *tropoelastin* will be used throughout the remainder of this discussion.

[28] L. B. Sandberg, N. Weissman, and D. W. Smith, *Biochemistry* **8**, 2940 (1969).
[29] D. W. Smith, D. M. Brown, and W. H. Carnes, *J. Biol. Chem.* **247**, 2427 (1972).
[30] J. A. Foster, C. B. Rich, S. Fletcher, S. R. Karr, and A. Przybyla, *Biochemistry* **19**, 857 (1980).
[31] S. R. Karr and J. A. Foster, *J. Biol. Chem.* **256**, 5946 (1981).
[32] J. M. Davidson, B. Leslie, T. Wolt, R. G. Crystal, and L. B. Sandberg, *Arch. Biochem. Biophys.* **218**, 31 (1982).

Extraction Methods

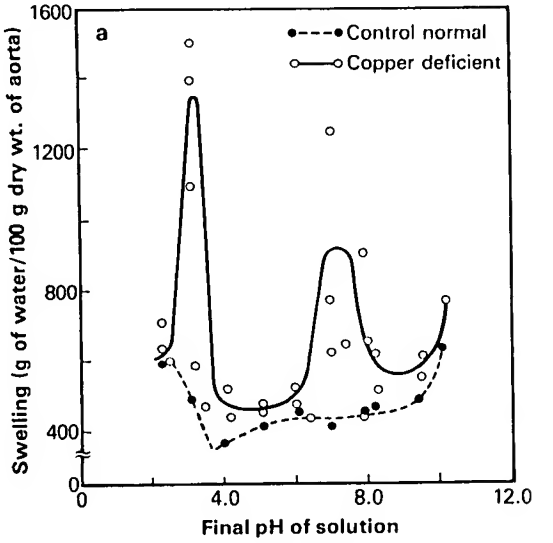
Guidelines for Purity. The amino acid composition of the two most important soluble forms of the protein is presented in Table I, that of the pig and the chick. These are average compositions and can be used as a guideline for assessment of purity taking into consideration that the "lesser" acidic and basic amino acid contents are not yet firmly established. It is to be noted that copper deficiency and lathyrism represent the only perturbations of normal biochemistry in these animals which will result in the yield of reasonable amounts of the protein from aortic tissue. Amino acid analysis is particularly useful in this protein as well for evaluation of purity because of the low levels of polar amino acids such as aspartate and arginine, as well as the absence of certain other amino acids such as methionine and histidine. A useful formula for rough calculation of the degree of contamination of "pure tropoelastin" or "pure elastin" utilizing the information from Table I is

$$\text{Contamination (\%)} = (\Sigma|\text{observed} - \text{expected}|)/10$$

The formula is applied to the 18 amino acids plus cross-links listed, and works well for contamination with proteins of "average" composition. When applied to collagen alone it somewhat underestimates the amount because of its high glycine and proline content but hydroxyproline in this case serves as a double check.

Swelling Properties of Elastic Tissue and Principles of Extraction. Figure 1a shows a swelling curve for copper-deficient porcine aorta in a low molarity buffered solution as might be used for an extraction solvent for tropoelastin.[33] The swelling curves demonstrate unique properties of copper-deficient elastic tissue which are important for tropoelastin isolation. Maximal swelling is seen at pH 3 and 7. Similarly, the extraction curves in varying salt molarities (Fig. 1b and c) indicate that the porcine aorta (or presumably any other copper-deficient elastic tissue) is likely to release more of its contents to the extraction buffer at low molarity pH 3 (Fig. 1b) or at 0.5 molarity and neutral pH (Fig. 1c). Thus a reasonable extraction buffer becomes ammonium acetate (which buffers at pH 7) at the 0.5 *M* level. Because of coacervation (an aggregative property of hydrophobic macromolecules) higher salt molarities at neutral pH are less desirable. Other suitable extraction solvents are 0.1 *N* acetic acid and 0.02 *N* formic acid (low pH and molarity).

[33] L. B. Sandberg, T. N. Hackett Jr., and W. H. Carnes, *Biochim. Biophys. Acta* **181**, 201 (1969).



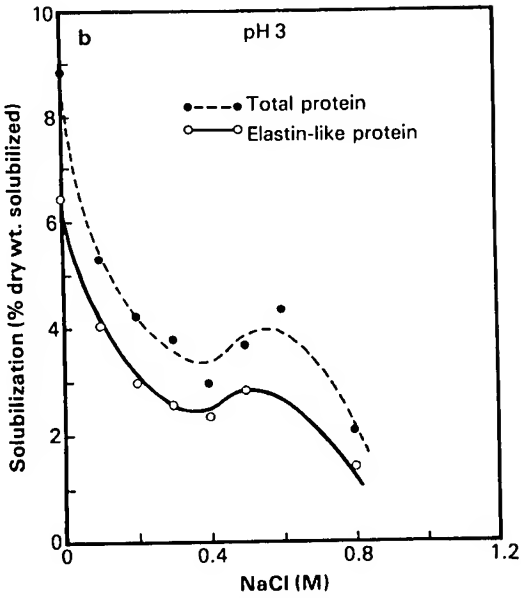


FIG. 1. Swelling of aortic tissues (a) from copper-deficient and normal young swine. Whole wet tissue portions (0.4–0.8 g) were swelled for 3 days in 50 ml of cold 0.15 *M* NaCl solutions buffered at the various pHs. Solubilization of proteins at pH 3 (b) and pH 7 (c) was evaluated as a function of salt concentration by recoveries of amino acids from hydrolyzates of 5% trichloroacetic acid precipitates. The content of elastin-like protein was calculated from the recovered valine content. Reprinted from Sandberg *et al.*[33] by permission of the publisher.

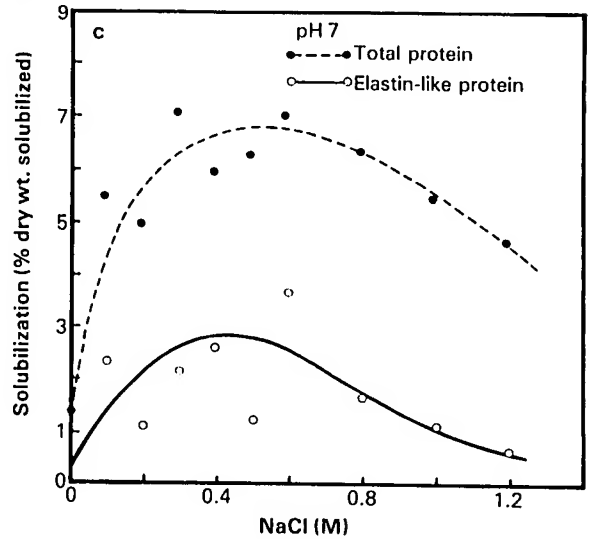


FIG. 1. (*continued*)

Use of Inhibitors. During early attempts at isolation of the protein little attention was paid to the use of protease inhibitors in the extraction medium. We soon learned, however, that inhibitors of serine proteases in particular are absolutely essential for isolation of the monomeric 70-kDa form of tropoelastin. Additional inhibitors are added in the form of *N*-ethylmaleimide (NEM) to inhibit sulfhydryl proteases, and EDTA, to inhibit metalloproteases, because tropoelastin and elastin have been demonstrated to have unusual sensitivity to all these classes of enzymes. The chick protein, which contains a tripeptide repeat, somewhat collagen-like,[34] is also susceptible to the tissue collagenases and thus attention to this susceptibility is also of utmost importance. Our extraction procedure therefore involves the following buffer and inhibitor mixture,[35] the data given in this series, Vol. 82 in [37] p. 661[8] being in error: 0.5 *M* ammonium acetate, 25 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* *N*-ethylmaleimide (NEM), and 1 m*M* diisopropylfluorophosphate (DIFP).

In addition, as indicated in Vol. 82 [37] pp. 661–664, DIFP must be added repeatedly to the extracted protein during the workup procedure particularly at times when the solution is allowed to stand for several hours. This is because of the presence of a slowly activating serine prote-

[34] D. W. Smith, L. B. Sandberg, B. H. Leslie, T. B. Wolt, S. T. Minton, B. Myers, and R. B. Rucker, *Biochem. Biophys. Res. Commun.* **103,** 880 (1981).

[35] L. B. Sandberg, E. Bruenger, and E. G. Cleary, *Anal. Biochem.* **64,** 249 (1975).

ase which copurifies with the tropoelastin.[36] Individuals who utilize dilute acetic or formic acid as an extraction solvent report the use of pepstatin as an effective protease inhibitor in that solvent.[37]

Methods of Purification of Soluble Elastin

Salt Precipitation and Coacervation. Although our initial extractions of tropoelastin were done in dilute formic acid,[28] we now use the neutral pH 0.5 *M* salt because the ammonium sulfate fractionation more quantitatively precipitates tropoelastin in this buffer.[38] Other extracted macromolecules such as collagen and glycoproteins are also precipitated with the ammonium sulfate.[38] By contrast, many serum-like proteins, including albumin, are soluble at this saturation and pH, thus affording a reasonable separation from them. Smith *et al.* separated collagen from tropoelastin by selective precipitation of the collagen from a 0.5 *M* NaCl, pH 7.2 extract of copper-deficient porcine aortic tissue (not unlike our 0.5 *M* ammonium acetate extract), through adjustment of the pH to 4.0 with 4 *N* acetic acid.[29] The collagen settles out as a flocculent precipitate removed by centrifugation. The tropoelastin can then be collected by heat precipitation at 37° from a 1 *M* NaCl solution at pH 8.0. This simple method is quite effective for high concentrations of tropoelastin (>1 mg/ml). Coacervation, however, is concentration dependent and lesser concentrations are much less efficiently precipitated.

Alcohol Fractionation. Details of the alcohol fractionation procedure are described by Sandberg and Wolt.[8] In most instances of isolation of tropoelastin from copper-deficient or lathyritic tissues, this method is to be preferred. The alcohol fractionation concept developed from a simple yet significant observation by Partridge that when alcohols are percolated through a gel filtration column made up of purified insoluble elastin swollen in dilute acetic acid, long-chain alcohols are retarded exceptionally well, far beyond a distribution coefficient of one, indicating that they are intimately involved in the internal structure of the elastin matrix.[39] Assuming that long-chain alcohols were exerting a solvent effect, we chose these same alcohols (butanol and propanol) to carry out an alcohol fractionation step which precipitates most other proteins but holds the tropoelastin in solution. The fractionation is efficient providing the tropoelastin concentration does not exceed 3–4 mg/ml in the ammonium formate buffered solution. For very low concentrations of tropoelastin

[36] R. P. Mecham and J. A. Foster, *Biochemistry* **16**, 3825 (1977).
[37] R. B. Rucker, this series, Vol. 82, p. 650.
[38] L. B. Sandberg, R. D. Zeikus, and I. M. Coltrain, *Biochim. Biophys. Acta* **236**, 542 (1971).
[39] S. M. Partridge, *Nature (London)* **213**, 1123 (1967).

(<0.1 mg/ml) the alcohol fraction step is not sufficiently specific to give a high degree of purity of the final product. Levels this low require HPLC or antibody affinity techniques for pure isolation (see summary discussion below).

Chromatographic Procedures. Several chromatographic procedures are useful in purification of tropoelastin. We have used gel permeation chromatography with Sephadex G-75 G-100, or G-150 in dilute formic acid as a final step after the alcohol fractionation procedure.[8] This affords some purification from minor low-molecular-weight contaminants as well as removal of salts. The procedure is not specific enough to afford any large degree purification. Exclusion chromatography on an anion-exchange resin has been used by some investigators taking advantage of the fact that the protein possesses virtually no negative charge and thus is poorly bound by the anionic resin under conditions which would bind virtually all other proteins.[37] Tropoelastin elutes at or near the column void with this system.

Weakly Acidic Carboxylic Cation Exchangers[28]

Materials

Amberlite IRC-50 (Rohm and Haas) or Bio-Rex 70 (Bio-Rad) 200–400 mesh
Column, monitor, collector, etc.
1 *M* potassium phosphate buffer, pH 7
0.01 *M* potassium phosphate buffer in 8 *M* urea, pH 7
0.1 *M* potassium phosphate buffer in 8 *M* urea, pH 7
1.0 *M* potassium phosphate buffer in 8 *M* urea, pH 7
0.02 *N* formic acid
(all neutral buffer molarities are calculated on the basis of potassium, and all contain 0.1% 2-mercaptoethanol)

Method. Ion-exchange chromatography is carried out at pH 7 in freshly deionized 8 *M* urea with 0.1% 2-mercaptoethanol buffered with potassium phosphate buffers. The resin is initially washed and equilibrated with a 1.0 *M* solution of potassium phosphate buffer without urea and stored in this fashion with a preservative such as thymol. At the time of use, a 10-cm column of the resin is poured with a cross-sectional area of 1 mm^2 per 5 mg protein to be applied. The column is pumped at a high flow rate, 1 ml/hr/mm^2 and at 8°, first with 0.01 *M* phosphate buffer in 8 *M* urea for equilibration, after which time the crude extract containing tropoelastin is applied having been dialized overnight against the same buffer. The column is then developed successively with 0.01 *M* (0.5 hr), 0.1 *M* (1 hr), and 1.0 *M* (1 hr) potassium phosphate in 8 *M* urea containing 0.1% 2-mercaptoethanol to protect the proteins from the effect of cyanate ion

produced by urea degradation. The pure tropoelastin elutes in the last fraction. It is dialyzed against 0.02 *N* formic acid containing 0.1% 2-mercaptoethanol.

Comment. Synthetic carboxylic acid cation exchangers will bind the tropoelastin sufficiently well, but not irreversibly, so that stepwise salt elution allows a significant purification. The procedure is most applicable to protein obtained from a dialyzed salt precipitate or a coacervate. Yields are not as good as those obtained from the reversed-phase method discussed below but the capacity is much higher

Reverse-phase C_8 HPLC

Materials

HPLC, monitor, collector, etc.
Aquapore column (Brownlee) RP 300, approximately 5 × 250 mm
Buffer A: 0.1% trifluoroacetic acid (Pierce) in water
Buffer B: 0.1% trifluoroacetic acid in 60% aqueous acetonitrile
Sample buffer: buffer A

Method. Figure 2 represents the parameters we have used by HPLC to achieve a purification. Running time is 80 min. The exact gradient conditions will vary for each individual column. The separation is done at ambient temperatures. The monomeric 70-kDa tropoelastin elutes in three peaks beginning at approximately 50% buffer B. The amount of protein distributed between the three peaks varies with each preparation. The first peak is accompanied by its degraded forms. The second and third peaks represent a more monomeric form of the protein but usually are lesser in amount. Finally its polymeric forms elute, some with the monomeric material and some after it. There is a distinct linear relationship with respect to elution times versus the molecular size of each eluted protein. A C_{18} guard column is always used.

Comment. It has been difficult to isolate monomeric tropoelastin efficiently from its degraded and polymeric forms. Although not ideal, this technique offers the purest product and is best applicable as a final purification step after the alcohol fractionation procedure. Maximum column capacity for good resolution appears to be about 10 mg.

Summary of Methods with Application

For trace amounts of tropoelastin, less than 100 μg, the protein is best purified by antibody affinity as described in the following chapter by Davidson and Sephel [11]. For small amounts, 10 μg to 10 mg, HPLC as described above is best suited. For larger amounts, 1 mg and above, the alcohol fractionation technique described in Vol. 82 of this series[8] is ap-

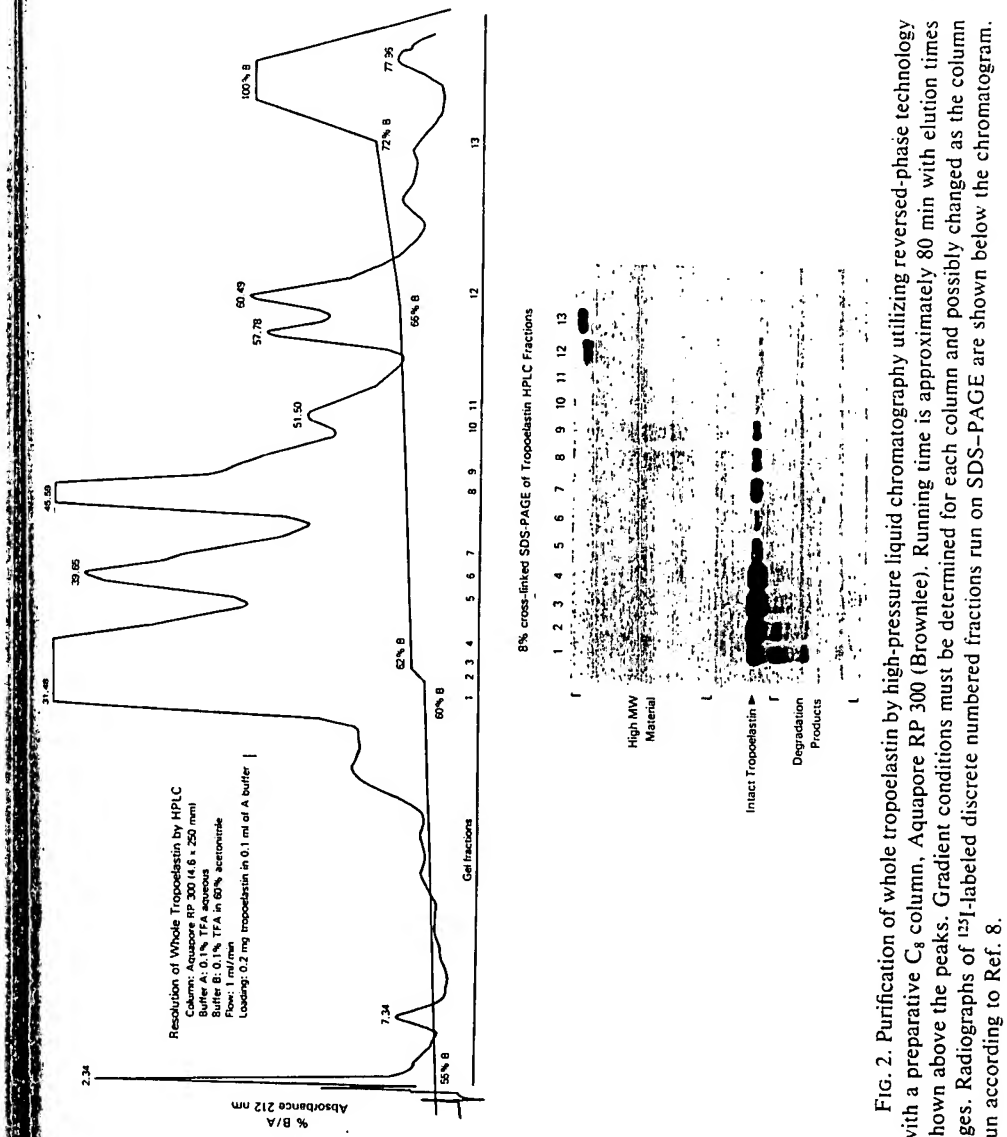


FIG. 2. Purification of whole tropoelastin by high-pressure liquid chromatography utilizing reversed-phase technology with a preparative C_8 column, Aquapore RP 300 (Brownlee). Running time is approximately 80 min with elution times shown above the peaks. Gradient conditions must be determined for each column and possibly changed as the column ages. Radiographs of ^{125}I-labeled discrete numbered fractions run on SDS–PAGE are shown below the chromatogram. run according to Ref. 8.

propriate. The carboxylic acid cation exchangers although they have a very high capacity appear best suited for isolation of elastin-associated proteins rather than tropoelastin itself. Quantitation of a sample or an aliquot above 5 μg is best achieved by amino acid analysis and below that amount by an ELISA assay directed against soluble elastin peptides.[40] Purity of product can be assessed visually by acrylamide gel electrophoresis as seen in Fig. 3, p. 663 of Vol. 82 of this series[8] or by amino acid analysis utilizing the formula discussed above or by Western blotting utilizing antibodies against α-elastin or tropoelastin as well as known contaminants. Amino acid analysis is the fastest and for us the most convenient method but Western blotting is the more sensitive.

Acknowledgments

This work has been supported by grants from the Veterans Administration. We are grateful to G. M. Giro,[40] who assisted in development of the SDS–PAGE patterns of tropoelastin seen in Fig. 2.

[40] M. G. Giro, K. E. Hill, L. B. Sandberg, and J. M. Davidson, *Collagen Rel. Res.* **4**, 21 (1984).

[11] Regulation of Elastin Synthesis in Organ and Cell Culture[1]

By JEFFREY M. DAVIDSON and GREGORY C. SEPHEL

Although elastin is an extremely insoluble protein polymer, cells synthesize the protein as a soluble precursor tropoelastin.[2–4] Production of this protein can be readily quantified by several methods, providing the cross-linking process is inhibited and steps are taken to reduce the exceptional proteolytic susceptibility of the precursor. Cross-link formation in elastin is mediated by the activity of lysyl oxidase, a copper-dependent enzyme which oxidatively deaminates ε-amino groups of lysyl residues in elastin and collagen. In elastin, some of these oxidized lysines condense into bi- and then tetrafunctional structures, the desmosines. Lathyrism, produced by β-aminopropionitrile or its analogs, is a means of inducing the abnormal but useful accumulation of the soluble elastin precursor by directly inhibiting the enzyme. Elastin synthesis is critical to the mechanical properties of numerous tissues: skin, lungs, arteries, vocal cords, and others. In addition, accumulation of elastin appears to be a hallmark of several pathologic conditions, including liver fibrosis, hypertension, breast tumors, and actinic damage to the skin.

Tropoelastin is an M_r = 70,000 monomer which is initially synthesized with a 26-residue signal sequence. It traverses the Golgi apparatus on its way from the rough endoplasmic reticulum to the cell surface, but there is no evidence for glycosylation of this protein. There appear to be two closely migrating forms of the newly synthesized protein on SDS–polyacrylamide gels,[5] but current evidence indicates they are derived from a single gene.[6] Recent DNA sequence analyses of the 3′ end of the gene and its cDNA have found two cysteinyl residues at the extreme COOH-terminus of the protein, which may imply interactions between tropoelastin molecules or between tropoelastin and other components of the elastic fiber.[7] The evidence for a larger precursor of tropoelastin (proelastin) is discounted by the characterization of the primary translation product of elastin mRNA and the size of the mRNA (3500 nucleotides, including 1200 nucleotides of 3′-untranslated sequence).

Developmental regulation of elastin synthesis has been the focus of many recent studies.[3] Most of the results point to a narrow window of intense elastin gene expression during middle to late fetal development. Under normal circumstances, elastin is not appreciably turned over during adult life, so that its deposition is a nearly irreversible event. The pathological destruction of elastin is discussed in a subsequent chapter.[8] In all the systems examined thus far, elastin production has been closely coupled to the steady-state concentration of elastin mRNA. Thus, in a

[1] Supported in part by the Veterans Administration, the Utah Heart Association, and NIH Grants GM32480, GM37387, and AG06528.

[2] L. B. Sandberg and J. M. Davidson, *in* "Peptide and Protein Reviews" (M. T. W. Hearn, ed.), Vol. 3, p. 169. Dekker, New York, 1984.

[3] J. M. Davidson and M. G. Giro, *in* "Regulation of Matrix Accumulation" (R. P. Mecham, ed.), p. 177. Academic Press, Orlando, Florida, 1986.

[4] J. M. Davidson, *in* "Connective Tissue Disease: Molecular Pathology of the Extracellular Matrix" (J. Uitto and A. Perejda, eds.), p. 29. Academic Press, Orlando, 1987.

[5] J. A. Foster, C. B. Rich, S. Fletcher, S. B. Karr, and A. Przybyla, *Biochemistry* **19**, 857 (1980).

[6] L. Olliver, J. M. Davidson, C. Mathew, P. A. LuValle, J. Rosenbloom, K. Yoon, A. J. Bester, and C. Boyd, *Collagen Rel. Res.*, in press.

[7] K. Yoon, J. M. Davidson, C. Boyd, M. May, P. LuValle, N. Ornstein-Goldstein, J. Smith, Z. Indik, A. Ross, E. Golub, and J. Rosenbloom, *Arch. Biochem. Biophys.* **241**, 684 (1985).

[8] M. J. Banda, Z. Werb, and J. H. McKerrow, this volume [15].

World J Urol (2000) 18: 19–25

Karl-Dietrich Sievert · Emil A. Tanagho

Organ-specific acellular matrix for reconstruction of the urinary tract

Abstract In urology, replacement of organs or organ segments has proved problematic. Current techniques do not replicate complete organ function, and they cause well-known complications. With the acellular organ-specific matrix we have found a way to regenerate tissue components seen in the normal lower urinary tract. The time required for regeneration depends on the matrix size and function. The matrix is covered by urothelium migrating from the host, after which neovascularization occurs, followed by formation of smooth-muscle cells and nerves. In our studies, normal muscle lining and nerves providing functional tissue were demonstrable and no sign of antigenicity was evident, even after heterologous grafting. The regenerated rat bladder was evaluated by organ bath as well as by in vivo functional tests and demonstrated properties and functions similar to those of host tissue. Besides our obtaining encouraging results in the rat bladder, we also studied the organ-specific acellular matrix in other species (dog and rabbit) and other organ segments (ureter and urethra).

Key words Homograft · Xenograft · Acellular matrix · Transplantation · Graft survival · Urodynamics · Organ bath · Growth factors · Animal models

Materials used to reconstruct the bladder must do more than replicate reservoir function [19]. Reconstruction must also address recurrent urinary tract infection, urolithiasis, renal parenchymal damage, and renal impairment and failure [24]. In the past, autologous bowel has been most commonly used for reconstruction, but absorption, mucous production, infection, stone formation, tumors, migration, and rejection have been described [32]. Material obtained at myomectomy or vesicomyectomy, covered with an omentum patch, has been used to extend bladder capacity in the sheep model, but inflammation and heterotopic calcification occurred and bladder function was not good [15]. Intestinal seromucosa has also failed. Even the placement of cultured urothelium on the graft has failed due to lack of adhesion [34].

Badylak's group has used a vascular graft made of matrix of small-intestine submucosa (SIS). In 1992 [44] they reported the first successful autogenous SIS graft for small-diameter arteries and, later, for the superior vena cava in the dog. Endothelium covered the lumen, and smooth-muscle cells were present throughout the depth of the transplant [4, 28, 29]. The biocompatibility of heterologous SIS has been demonstrated by a patch augmentation of the pig bladder using bovine material with no inflammatory reaction. Ingrowth of capillaries and smooth muscle seen in later specimens suggested that it could be used for a segment of the dog bladder [25].

In our studies we have found that a full-thickness, homologous-bladder acellular matrix graft (BAMG) closely matches the host tissue with regard to size and to mechanical, structural, and genetic properties [42]. After successful homologous grafting [39] a heterologous BAMG (prepared from hamster, rabbit, or dog tissue; see Fig. 1A, B) was used to augment > 50% of the resected bladder in male and female rats. Biochemical analyses confirmed the retention of collagen and elastin and some extracts of glycosaminoglycans in the acellular specimens, and Piechota et al. [39] obtained a matrix completely free of recognizable cellular elements.

Following the successful morphologic and functional regeneration of detrusor smooth muscle in the rat model

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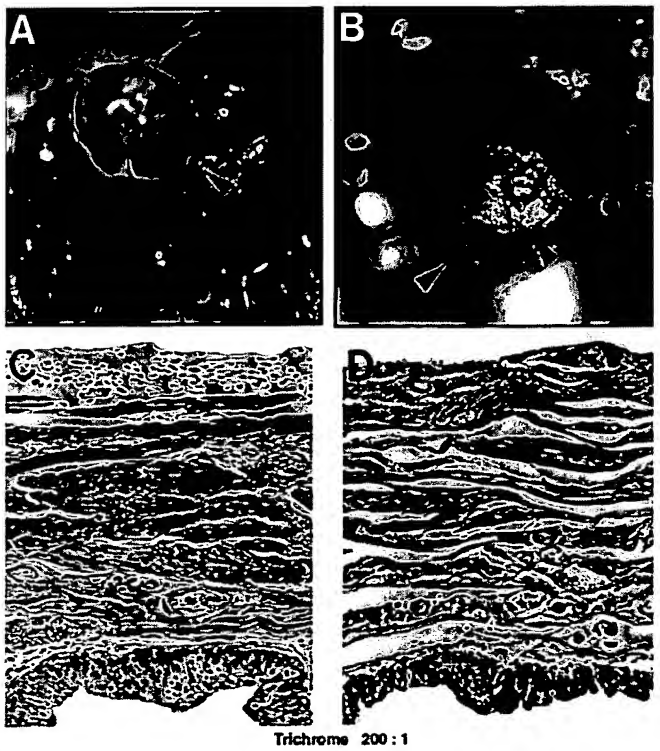

Fig. 1A–D Bladder acellular matrix graft prepared from hamster tissue as evaluated in the rat model. Macroscopic appearance at surgery (A) and 4 months later (B). Note that the regenerated segment is identifiable only by the marking sutures. Histologic appearance of the host tissue (C) and matrix (D) at 4 months. Note that all three layers of normal bladder wall are present

the study was extended to elucidate the mechanisms of functional innervation of the BAMG in vivo. Furthermore, in vitro electrical and pharmacologic stimulation techniques were used to study contractility and to characterize the expression of neurotransmitter receptors [40], and transforming growth factors were examined by reverse-transcription polymerase chain reaction [13].

Materials and methods

Preparation of the acellular matrix graft

Preparation of the graft was carried out as described by Piechota et al. [40] and modified from the method of Meezan et al. [33]. Tissue (bladder, ureter, or urethra) obtained through our institution's tissue-sharing program was harvested. The tissue (used in most studies without mechanical scraping of the epithelium) was placed in 10 m*M* phosphate-buffered saline (PBS, pH 7.0) and 1% sodium azide and was stirred for several hours for partial cell lysis, the duration depending on the tissue size and thickness. Cell lysis was completed in 1 *M* sodium chloride containing 2000 Kunitz units of DNAse (Sigma, St. Louis, Mo., USA) and the mixture was stirred again. Specimens were treated twice in 50 ml of 4% sodium desoxycholate containing 0.1% sodium azide and were stirred for 5–6 h for solubilization of the lipid membranes. The resultant acellular matrix was washed three times in PBS and stored in 10% neomycin sulfate at 4 °C until grafted.

Surgical technique

The Sprague-Dawley rat was the first model examined [13, 39–42]. Through a midline incision the catheterized and filled bladder was exposed in the anesthetized rat (pentobarbital 40 mg/kg). The previously prepared and trimmed BAMG was sutured onto the bladder (after hemicystectomy) with four marking sutures (Dexon 7.0); the four sides were then continuously sutured with absorbable material (Dermalon 7.0). The graft-host anastomosis was checked for leakage by filling of the bladder through the transurethrally placed 3-Fr catheter (CR Bard Inc., Covington, Ga., USA), and full capacity at surgery was noted. Finally, the abdominal wall was closed in layers.

In a second study, a 0.3- to 0.8-cm section of rat ureter was excised and replaced with an acellular graft of equal size placed on a polyurethane stent. The graft was anastomosed end to end with monofilament nonabsorbable suture (Dermalon 10.0). For the promotion of growth the matrix was surrounded with retroperitoneal fat before the abdominal wall was closed in layers [12].

In the dog bladder we used the same technique applied in the rat, and the bladder was replaced with a homologous [43] or heterologous BAMG (Sievert et al., manuscript in preparation). Oral antibiotics were given until the transurethral catheter was removed.

In the male New Zealand rabbit the pendulous urethra was replaced by an acellular urethral homograft [46] and heterograft [47]. Through a ventral midline penile-skin incision, tissue was mobilized from the corpora cavernosa. A segment of about 1.0 cm was excised and replaced with an acellular urethral tube graft. The graft was sutured in place end to end with interrupted absorbable 7–0 suture and was marked by three nonabsorbable 7–0 sutures. The wound was closed in layers using absorbable sutures.

In vivo functional studies

Bladder function was evaluated by cystometry after 4 months in most studies [13, 41]. In addition, the pelvic nerve was stimulated in animals under urethane anesthesia (1.2 g/kg given s.c.). The effect of the electrostimulation was assessed before and after i.v. administration of atropine sulfate (0.5 mg/kg) or hexamethonium bromide (5 mg/kg; a nondepolarizing ganglion-blocking agent [52]) or topical lidocaine application (0.2 ml, 1%).

In urethral studies, after urethral pressure-profile and urodynamics investigations [46] the replacement was evaluated by retrograde urethrography before surgery and at euthanasia, up to 8 months thereafter.

Electrical field stimulation

For functional studies, full-thickness longitudinal bladder strips of uniform size (approx. 2×7 mm^2) were obtained from the host and the regenerated BAMGs. The strips were stimulated in a tissue bath (described in detail by Piechota et al. [40]) and the results of electrical field and pharmacologic stimulation were recorded [40].

Reverse-transcription polymerase chain reaction

Total extracted RNA was determined in homologous BAMG-regenerated tissue at 2 weeks as well as 1, 4, and 6 months after grafting. Through reverse-transcription polymerase chain reaction (RT-PCR) the samples were examined for the expression of specific growth factors (transforming growth factor: TGF-α, TGF-β_1, TGF-β_2, and TGF-β_3) that appear to play a key role in bladder development [5].

In urethral studies the RNA of normal and regenerated urethral tissue (specimens from day 10, weeks 3 and 6, and months 3, 6, and 8) was analyzed for insulin-like growth factor (IGF) and heparin-binding growth factor (HB-EGF) as well as for TGF-α, TGF-β_1, and β-actin [46].

Staining

Acellular specimens were embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) for cell nuclei. In addition, several BAMG specimens (rat, hamster, dog, pig, and human [39, 40]) were examined by electron microscopy and Hart's elastin and picrosirius acid-collagen staining (rat, pig, and human [14]).

For evaluation of regeneration the excised tissue was fixed in formalin, embedded in paraffin, sectioned, and stained for collagen and smooth muscle (Masson's trichrome; Fig. 1C, D), α-actin (smooth muscle), and nonspecific neuronal protein gene product (PGP 9.5). After 4 and 8.5 months, differential nerve staining for acetylcholinesterase (AChE) [16], adrenergic nerves [54], and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase [50] (nonadrenergic, noncholinergic nerves) was conducted in addition to staining for PGP 9.5 [40]. For urethral replacement, acellular urethral tissue and regenerated tissue were stained as described for the BAMG (except for PGP 9.5).

Results

Histologic and immunohistochemical examination

In the acellular matrix of the different species, collagen fibers, although not quantified, appeared to be closely packed, with variations in diameter being observed in the rat, pig, and, particularly, human matrix [14]. Elastic fibers were seen in all species, and histologic differences were apparent in both elastin and collagen fibers; picrosirius acid-stained specimens showed type I to be the major collagen fiber for the rat BAMG, in contrast to the pig and human BAMG, where collagen type III was abundant. On scanning and transmission electron microscopy the structure of the acellular matrix scaffold varied in the different species, but it was not possible to distinguish distinct collagen types [14, 39].

For evaluation of acellular tissue regeneration, sections were examined at various time points after surgery. The luminal surface of the matrix was half covered by mucosa (urothelium) after 1 week. Red blood cells and mononuclear cells infiltrated the host-matrix anastomosis. α-Actin staining demonstrated involvement of the first positive smooth-muscle cells, not well oriented, in the formation of vessels. Within 4–6 weeks postoperatively, several layers of urothelium covered the complete matrix more or less uniformly, showing no difference from host urothelium. The first vessels were oriented parallel to the urothelium, and muscularization had begun. PGP 9.5-positive staining (nerves) was initially picked up after 4 weeks. With time (12 weeks) the amount of smooth muscle increased up to 36% in the acellular matrix (the proportion of smooth-muscle cells displayed by the host was 44%; comparative photoanalysis using PhotoShop 3.0), and the border of the anastamosis was sometimes difficult to identify. The muscle cells were oriented and the number of vessels as well as the diameter of nerves had increased [42]. In the 4-month specimen the thickness of the muscle bundles seemed to decrease toward the center of the graft, whereas revascularization appeared to be equally pronounced at the edges and in the middle of the homologous BAMG [40]. Although these features were not evaluated quantitatively, light microscopy of the heterologous findings was consistent [39] with the results recorded for rat-BAMG differentiation over time (Fig. 1C, D) [42].

After 8.5 months the homologous BAMG had a more balanced dispersion of vessels and muscle fibers throughout the graft than was evident at earlier time points. The α-actin-positive muscle fibers seemed to be stable and the number of vessels seemed to have decreased as compared with the 4-month findings. PGP 9.5-positive nerve fibers were seen predominantly next to muscle bundles and the luminal surface of the graft [40]. There was no sign of antigenicity or scar tissue [13, 39–42].

In the ureteral graft, all specimens revealed varying degrees of hydroureteronephrosis macroscopically at the level of the matrix. However, regeneration was temporally similar to that observed for the BAMG. At month 3, PGP 9.5 was first detected in the graft, and within another month, neomuscularization was well developed. The smooth-muscle cells were arranged longitudinally, with decreased numbers occurring toward the center of the graft. The urothelial lining and muscularization appeared to be similar to those seen in normal (host) ureteral wall (confirmed by transmission electron microscopy) [13].

In the canine model, homologous BAMG regeneration took up to 7 months and smooth-muscle hyperplasia was not seen (as it had been in the control group [42]).

The smooth-muscle cells in the urethral replacement in the rabbit were not as orderly as those in the host after 6 months, although all components were seen at 3 months [46, 47].

Cystometric and electrophysiologic evaluation

At 4 months postoperatively, both the capacity and the residual volume of the BAMG-grafted bladders were significantly higher than those observed in normal rats (2.05 ± 0.28 versus 1.23 ± 0.02 ml and 1.49 ± 0.22 versus 0.74 ± 0.13 ml, respectively) [13]. In the heterologously grafted animals the capacity was similar to that seen in the homologously grafted animals after 4 months (hamster 2.49 ± 0.04 ml, dog 2.23 ± 0.09 ml, rabbit 2.08 ± 0.04 ml) [39]. No significant difference in micturition pressure was found between the groups. The leak-point pressure found in grafted animals did not differ significantly from that measured in controls. Compliance in the latter was significantly decreased [39].

In rats that received a heterologous BAMG, voiding volumes were < 50% of the values noted for controls after 3 days; however, at the end of 4 months, voiding volumes exceeded the preoperative values in the grafted and control animals by 72–94% and 35%, respectively.

In the grafted animals, pelvic nerve electrostimulation produced 50% of the bladder pressure of controls at 4 months.

In animals that underwent urethral grafting, neither urodynamics data nor urethral pressure profiles differed from control values after 8 months [47]. Retrograde urethrography demonstrated extravasation at about day 10 and hyperflexibility up to the 3rd month. A very good adaptation to the host urethra was demonstrated from month 6 onward, making it nearly impossible to detect the anastomosis on X-ray [46, 47].

Electrical field stimulation

When BAMG-regenerated strips of homologously grafted animals were compared with normal bladder strips the response to supramaximal electrical field stimulation was quantitatively similar; the maximal force of contraction of the regenerated tissue amounted to 62% of the host bladder-wall tissue after 4 months [40]. As compared with the appropriate host bladder-tissue strips, the maximal contraction force was almost the same in all heterologously augmented groups (dog BAMG 85%, hamster BAMG 42%, rabbit BAMG 38% [39]).

Organ bath

The muscarinic response, tested by cumulative addition of carbachol, was qualitatively identical, but at the peak contractions measured at 10^{-4} *M* the regenerated strip reached 79% of the host bladder-strip response after 4 months [40]. For the heterologously grafted bladder the maximal force of contraction was similar in all BAMG-regenerated strips (dog BAMG 60%, hamster BAMG 50%, rabbit BAMG 34% as compared with the appropriate host bladder-tissue strip) and was double that determined following electrical stimulation [39]. Atropine (1×10^{-6} *M*) and valethamate bromide (2.6×10^{-5} *M*) completely relaxed all carbachol-contracted strips and enhanced spontaneous muscle contraction for 6 and 5 min, respectively [40]. In the heterologously grafted animals the BAMG-regenerated carbachol-contracted strips relaxed following treatment with atropine to a level below their initial resting tension [39].

Potassium (60 m*M*) evoked sustained contractions in the homologous regenerate that amounted to 32% of those measured in host bladder-wall tissue [40]; in heterologous regenerates the corresponding results were 86% in dog BAMG, 44% in rabbit BAMG, and 29% in hamster BAMG (as compared with contractions determined in host-bladder smooth-muscle strips) [39]. The nonadrenergic compound nitroprusside (10^{-4} *M*) relaxed the potassium-induced contraction of both strip types to the same extent.

Reverse-transcription polymerase chain reaction

Unlike amounts of TGF-β_2 and TGF-β_3, levels of TGF-β_1 and TGF-α were significantly increased from week 2 to month 6, with a prominent message level being determined for TGF-α at month 6 [13]. In contrast to previous work in the rat model, rabbit tissue showed an increase over time in IGF and HB-EGF. IGF levels increased by week 3 and returned to normal around month 6; HB-EGF was not detected until day 10 postoperatively but was up-regulated at 3 weeks and was expressed until month 6 [46].

Stones

Of the partially cystectomized (control) animals, 29% developed stones, whereas almost 85% of grafted animals had urinary calculi. However, the stone size and composition did not differ (struvite 60–100%, apatite 40–100%, newberrylite 20–100%, brushite 10–100%) [39].

Discussion

Organ reconstruction plays an essential role in the surgical field. In the urinary tract, bowel has been the most common material used to date, with well-documented urodynamic benefits [44]. Although autologous, this grafted tissue cannot replicate all the necessary functions of the urinary tract, and absorption and mucous production have remained problematic. In addition, gastrointestinal-tract complications have been well documented [7, 18, 20, 24, 32, 35, 45].

Gleeson and Griffith [19] have defined the ideal material as being biocompatible and mechanically reliable, resistant to extraluminal infection but tolerant of intraluminal infection, and easy to implant surgically. For the bladder it should preserve renal function, provide adequate urinary storage at low pressure, and allow volitional, complete evacuation of the bladder per urethram.

Previous studies using tissue of small-intestine submucosa, processed by a technique of Meezan et al. [33], resulted in an acellular collagen and elastin scaffold [3, 4] with the potential to regenerate and provide functional capacity. Because of these encouraging results, over the past 5 years we have undertaken studies using an organ-specific acellular matrix, which has been shown to be successful for partial bladder and ureteral replacement [12, 13, 39–42].

Functional changes with time confirmed the autoregeneration of the BAMG. Micturition pressure did not vary significantly, and the increased capacity reflected new muscularization and innervation in the graft [13]. As in the rat model, Probst et al. [43] reported a significant increase in bladder capacity after BAMG in the dog model after 7 months. In contrast to the results obtained by Dahms et al. [13], whereby unilateral pelvic

nerve stimulation did not provide adequate contraction and increased intravesical pressure, we found that bilateral sacral root stimulation enabled us to increase the bladder pressure above the sphincteric pressure measured in dogs that had received a heterologous BAMG, with voiding being the result (Sievert et al., manuscript in preparation). Although urethral function is not as complex as bladder function, urethral replacement by the acellular matrix demonstrated complete function on retrograde urethrography [46, 47].

In our studies the mortality secondary to suture-line leakage was 15% overall, consistent with rates reported for similar operations in the rat [26, 28, 49]. In our recent canine studies, none of the animals was lost ([43]; Sievert et al., manuscript in preparation). In our rabbit-urethra studies, none of the animals died after homologous or heterologous grafting [46, 47]. Indeed, in no study did an animal die of causes related to immune response. Cross sections of BAMGs and urethral replacement showed only a trivial number of leukocytes and lymphocytes, thus indicating no sign of rejection [27].

Bladder stone formation, which is common after surgery in the lower urinary tract of the rat [30, 31], was seen in our rodent studies [42, 49] but was not seen in the canine model for up to 7 months after transplantation ([43]; Sievert et al., manuscript in preparation). Because struvite was the main component, infection was the most likely cause [21].

Over a continuous follow-up period of up to 8.5 months, in the BAMG as well as in the ureteral acellular matrix graft (UAMG) we demonstrated regeneration of urothelium, vessels, smooth muscle, and nerves in the rat [12, 39]. Over the first few days there was rapid epithelialization of the surface, which soon became a multilayered normal urothelium. In the same way, neoangiogenesis from the host tissue into the scaffold took place. On the other hand, we hypothesize that smooth-muscle cells might differentiate from myofibroblasts, which are very similar in ultrastructure [48], a hypothesis supported by other recent reports [17, 57].

Using electrical field stimulation of intrinsic nerves, Piechota et al. [39, 40] demonstrated the contractility of the BAMG-regenerated tissue and host bladder wall. They showed that PGP 9.5-positive nerves had reinnervated the muscle cells; tetrodotoxin, which abolishes neurogenesis action potentials, provided further evidence of neuronal components.

The presence of muscarinic, adrenergic, and purinergic receptors [9, 10, 22, 23, 55] was shown through the use of several agonists and antagonists in the host as well as in the BAMG regenerates and was additionally confirmed by histochemical staining for cholinergic and adrenergic nerves [40]. Because of a comparable tissue-bath reaction shown by heterologous BAMG regenerates and control tissue [39], the presence of adrenergic and cholinergic nerves can be assumed.

Potassium induced the contraction of regenerated BAMG and host-bladder smooth-muscle cells [39, 40] by direct depolarization of membrane potential, supporting the probable involvement of a neuronal nitric-oxide-containing nerve pathway in nonadrenergic, noncholinergic relaxation [2, 38]. The organ-bath findings were also supportive, as the contractility of the BAMG-regenerated strips was about half that of the host-bladder strips [39].

Baskin et al. [5, 6] were the first investigators to report the influence of growth factors on bladder development. In our studies, levels of two of four growth factors (TGF-α and TGF-β_1) were increased over time in BAMG regenerates [13], and other workers have found increased amounts of TGF-α protein after iatrogenic bladder manipulation [11]. The family of TGF-βs can inhibit or induce the proliferation of different cells [36]. TGF-β_1 has been characterized as making smooth-muscle cells less capable of migrating and more adherent [51] and rendering certain smooth-muscle cell types growth-inhibitory [53]. In our rabbit-urethra model [46], TGF-β_1 was detected, but the levels were equal in normal and grafted tissue. Further study demonstrated that, after 10 days, levels of IGF increased significantly for up to 6 months postoperatively. Hyperplasia of smooth-muscle cells was seen with increasing levels of IGF [56], but IGF may be one of the growth factors responsible for the development of smooth-muscle cells and may be down-regulated before hyperplasia occurs. Heparin-binding epidermal growth factor (HB-EGF, which stimulates the growth of fibroblasts and smooth-muscle cells [37], promotes neovascularization, induces endothelial cell migration in the bovine model [1], and regulates urothelial proliferation [37]) was not detected in normal rabbit urethral tissue but increased after day 10 and decreased at month 6 in the regenerates. Recently, the presence of HB-EGF and HB-EGF-like growth factor (erbB-1, erbB-2) in human urothelium, bladder detrusor, and smooth muscle was reported [8].

Using different animal models, we have been capable of demonstrating an inexpensive method for the production of a scaffold for partial regeneration of urologic organs. With increased knowledge of both the mechanisms underlying regeneration with this organ-specific acellular matrix and the importance of certain growth factors, we will attempt to shorten the time required for regeneration and to increase the number of smooth-muscle cells for better function.

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ELSEVIER

FREE URETERAL REPLACEMENT IN RATS: REGENERATION OF URETERAL WALL COMPONENTS IN THE ACELLULAR MATRIX GRAFT

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ABSTRACT

Objectives. To evaluate ureteral replacement by a free homologous graft of acellular matrix in a rat model.
Methods. In 30 male Sprague-Dawley rats, a 0.3 to 0.8-cm midsegment of the left ureter was resected and replaced with an acellular matrix graft of equal length placed on a polyethylene stent. The animals were killed at varying intervals, and the grafted specimens were prepared for light and electron microscopy.
Results. In all animals, the acellular matrix graft remained in its original position without evidence of incrustation or infection, and histologic examination showed complete epithelialization and progressive infiltration by vessels. At 10 weeks, smooth muscle fibers were observed; at 12 weeks, nerve fibers were first detected; at 4 months, smooth muscle cells had assumed regular configuration.
Conclusions. The ureteral acellular matrix graft appears to promote the regeneration of all ureteral wall components. UROLOGY **50:** 818–825, 1997.

Ureteral replacement has long been a subject of interest to researchers, and many surgical procedures and materials have been tried.[1–4] However, an entirely satisfactory method has yet to be found. Previous research has demonstrated that collagen-based materials, such as porcine small intestine submucosa (SIS), have the best potential regenerative capability.[5] In this group of biomaterials, a new acellular matrix has recently been shown, in the bladder of the rat model, to serve as a scaffold consisting of collagen and elastin fibers for the ingrowth of all bladder wall components.[6,7] In addition, the contractility of graft-regenerated bladders has been observed in vivo (preliminary results). We designed the present study to determine whether this acellular matrix could be used as a free ureteral graft in a rat model.

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MATERIAL AND METHODS

Animals and Surgical Technique

Male Sprague-Dawley rats (n = 30), weighing 260 to 360 g, were housed three to a cage at constant temperature and humidity with a 12-hour light and dark cycle. Rats were given free access to standard labaratory chow and tap water. The technique for preparation of the matrix graft, described previously for the bladder,[7] was modified as follows: the ureter from a Sprague-Dawley rat was excised and placed on a polyethelene stent (inner diameter [ID] 0.28 mm; outer diameter [OD] 0.61 mm) for further processing. Because the tiny rat ureter is not suitable for inversion, for the ureteral matrix the mucosa was not scraped off as it was in the preparation of the bladder matrix graft. Partial cell lysis (in 10 mM PBS), complete cell lysis (in 1 M sodium chloride containing 2000 Kunitz units DNase), and solubilizing of the lipid membranes in 50 mL of 4% sodium desoxycholate containing 0.1% sodium azide (repeated once) were performed. The resulting ureteral acellular matrix graft was stored in 10% neomycin sulfate at 4°C until grafted (Fig. 1).

At the time of surgery, animals were anesthetized with sodium pentobarbital (6 mg/100 g body weight intraperitoneally) and, through a midline lower abdominal incision, the left ureter was exposed. A 0.3 to 0.8-cm midsegment of the ureter was resected and replaced with an equal graft segment placed on a polyethylene stent through the open ends of the host ureter (Fig. 2). The stent was used to prevent obstruction at the level of the graft. The graft segment was trimmed to conform to the remaining host ureter and sutured in place with monofilament 10.0 nonabsorbable Dermalon sutures to identify the matrix borders. To promote growth, the acellular matrix graft was surrounded with retroperitoneal fat. When satisfactory closure was achieved, the abdominal wall

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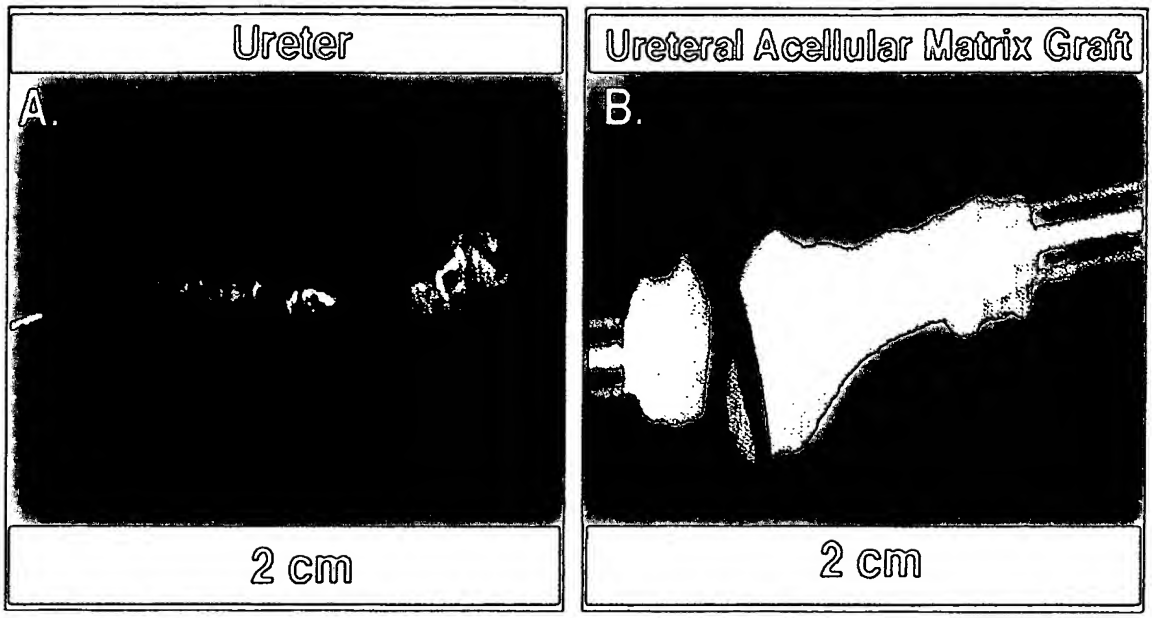


FIGURE 1. *Photographic documentation of a rat ureter placed on a polyethylene stent before (A) and after (B) processing into a ureteral acellular matrix graft.*

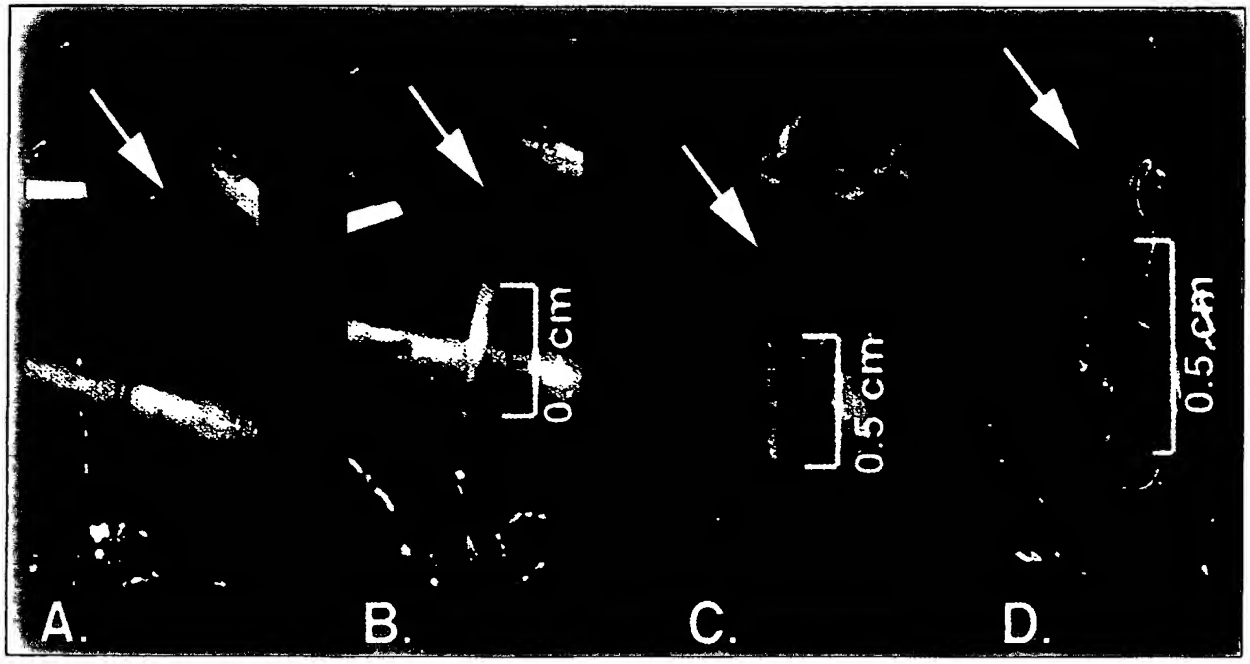


FIGURE 2. *Surgical procedure of partial ureteral replacement by the ureteral acellular matrix graft: (A) exposure and temporary clamping of the left ureter; (B) ureterotomy and intubation by the stented graft in the direction of the urinary bladder (arrows); (C) distal anastomosis with interrupted sutures; (D) intraoperative view after implantation.*

and the skin were closed. For microsurgery, an Olympus binocular operating microscope (10× to 40×) was used. No drugs were administered postoperatively.

Light Microscopic and Ultrastructural Evaluation

The animals were killed at the following times: 4 days (n = 3), 3 weeks (n = 5), 6 weeks (n = 3), 10 weeks (n = 7), 3 months (n = 5), and 4 months (n = 5). Host ureter and graft were identified, freed from the surrounding tissue under a dissecting microscope, and collected. All specimens were rinsed with saline solution to remove excess intraluminal urine. Tissues were fixed at the time of accession and processed for light and transmission electron microscopy.

Light Microscopy. Specimens were fixed in 10% buffered formalin for at least 24 hours. After dehydration in graded ethanol solutions, the specimens were embedded in paraffin, sectioned (5 μm), and stained with trichrome for collagen and smooth muscle, hematoxylin and eosin (H & E) for nuclei, alpha-actin for smooth muscle, and protein gene prod-

FIGURE 3. *Confirmation of the acellularity of the matrix graft and its structure as a framework of elastin and collagen fibers: (A) H & E staining at an original magnification of ×200; (B) scanning electron microscopy at low magnification (original magnification ×25) shows the intact surface of the ureteral acellular matrix; (C) at higher magnification (original magnification ×130), the mesh-like structure of fibers (which may serve as a scaffold), without evidence of cellular elements, is demonstrated.*

uct 9.5 (PGP 9.5) for nerves. The monoclonal antibody anti-alpha-smooth muscle actin recognizes alpha-smooth musle cells exclusively.[8] Protein gene product 9.5 represents a major protein component of the neural cytoplasm and therefore labels more nerve fibers than other general nerve markers.[9,10] Before implantation, pure graft specimens were also prepared for light and scanning electron microscopy to confirm the structure as an acellular scaffold consisting of collagen and elastin fibers and thus the effectiveness of the matrix preparation process.

Transmission Electron Microscopy. Specimens were immersed in a fixative (2.5% glutaraldehyde and 2.5% paraformaldehyde) in 0.15 M sodium cacodylate buffer. After the primary fixation, the samples were placed in a drop of fixative on dental wax and cut in approximately 3-mm segments. Specimens were postfixed with 2% osmium tetroxide, block stained in 2% uranyl acetate, and dehydrated in a graded series of ethanol and propylene oxide, after which they were embedded in resin. Thin sections (500 Å) were obtained, mounted on 200-mesh copper grids, stained in uranyl acetate and lead citrate, and examined in a Zeiss Model 10c electron microscope.

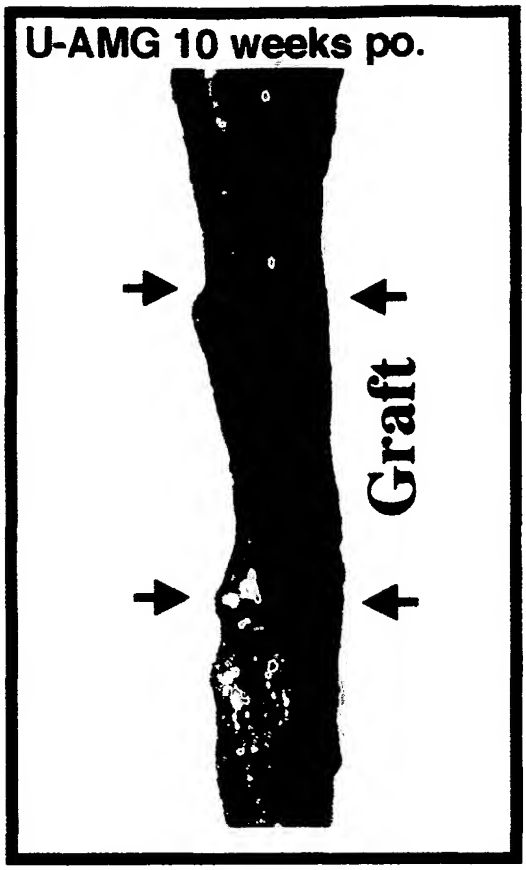


FIGURE 4. *Macroscopic view of the rat ureteral matrix graft 10 weeks after surgery. Arrows mark nonabsorbable sutures to identify the border between the matrix graft and host.*

RESULTS

MORTALITY

Of the 30 animals, 2 died 6 weeks after surgery from a coronavirus infection associated with severe respiratory tract obstruction and were not used for further microscopic evaluation. In contrast to our reported experience with the matrix grafted to the bladder,[7] stone formation was not observed in either the upper or lower urinary tract.

EVALUATION OF MATRIX TECHNIQUE

Light microscopy of the pure ureteral matrix demonstrated the effectiveness of the matrix preparation process. The acellularity of the graft as an intact framework consisting of elastin and collagen fibers was apparent (Fig. 3A). Scanning electron microscopy showed the intact nature of the ure-

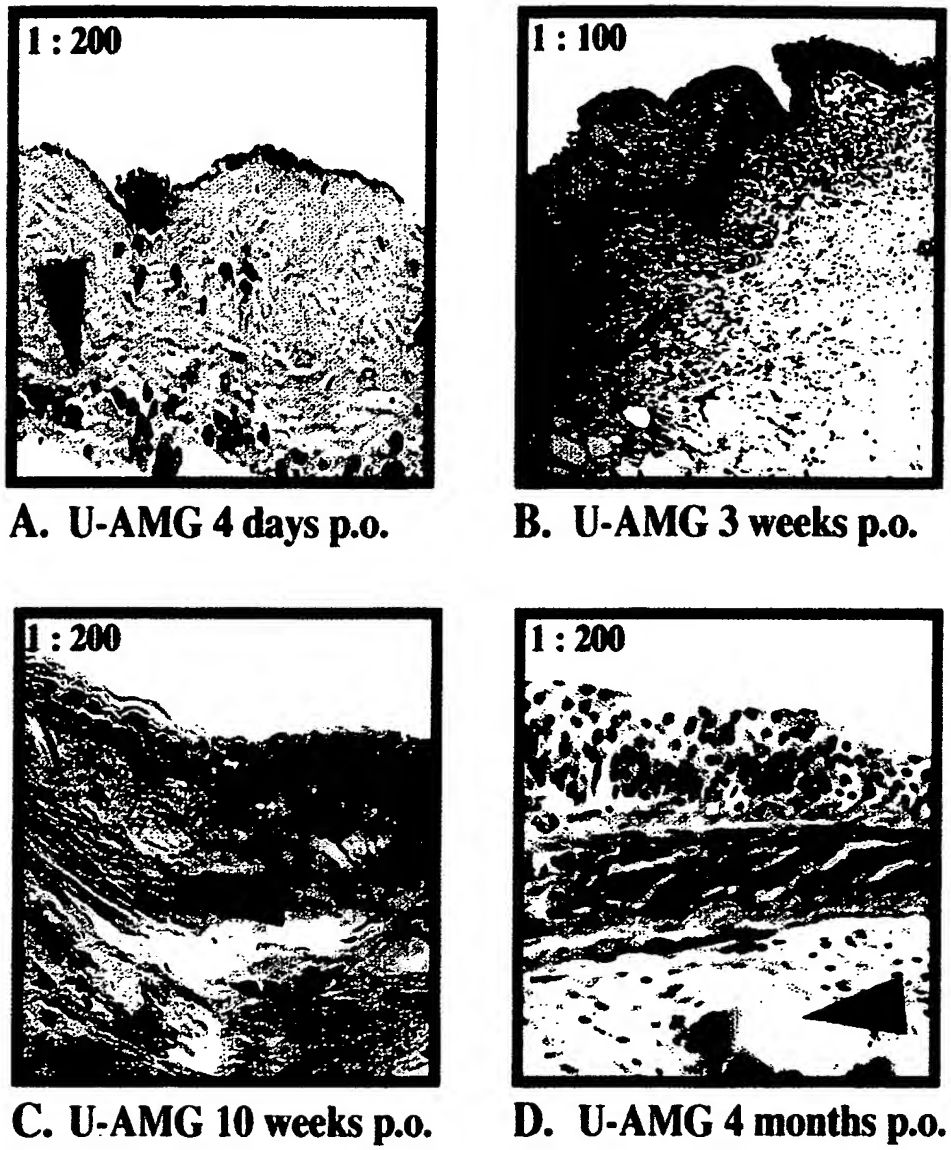


FIGURE 5. *Step-by-step regeneration of ureteral wall components (Masson's trichrome stain, original magnification ×200). After infiltration of erythrocytes and mononuclear cells (arrow) (A), urothelium (arrow) begins to develop in the first weeks (B). At 10 weeks (C), the first few muscle fibers are seen (arrow), and after 4 months (D), a complete regeneration of urothelium and muscularization can be observed (arrow marks borderline [suture] between the matrix graft [left] and the host ureter [right]) (U-AMG, ureteral acellular matrix graft).*

teral matrix surface and confirmed the scaffold-like structure of the graft without evidence of cellular elements (Fig. 3B, C).

Macroscopic Appearance of Explanted Grafts

Moderate adhesions were noted to the surrounding retroperitoneal fat. The graft remained in its original position in all animals, without evidence of incrustation or infection (Fig. 4), whereas the polyethylene tube migrated to the distal ureter. For this reason, gross examination of all surgical specimens revealed varying degrees of hydroureteronephrosis to the level of the graft. There was no evidence of postoperative urinary leakage in any of the animals at death.

Light Microscopy

Histologic examination of all graft-regenerated ureters showed step-by-step regeneration of urothelium and smooth muscle fibers (Fig. 5). At 4 days, the graft showed an infiltration of erythrocytes and mononuclear cells, and the urothelial lining appeared to begin to bridge the matrix graft. At 10 weeks, the graft was composed of several layers of urothelium and some capillaries, and characteristic arrangements of smooth muscle fibers (Fig. 6) were first observed. The number of vascular elements (capillaries) had increased. At 12 weeks, nerve regeneration was first detected by PGP 9.5-positive staining (Fig. 7). At 4 months, neomuscularization was well developed. The

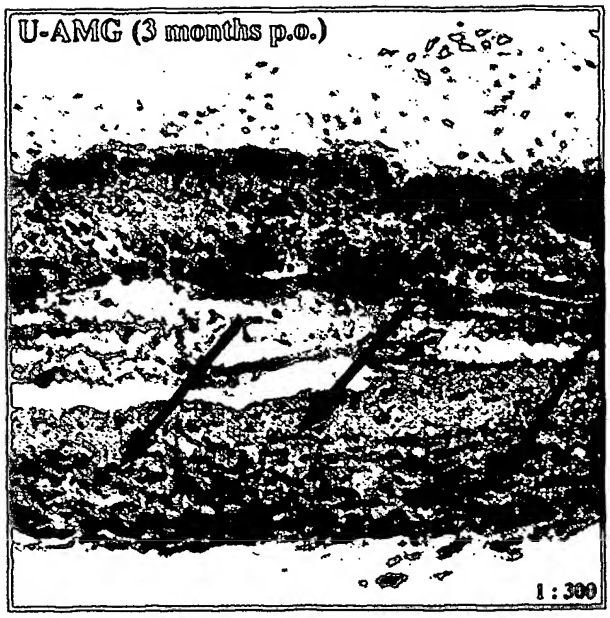


FIGURE 6. *Histologic appearance of the ureteral acellular matrix graft (U-AMG) 3 months after grafting. Smooth muscle cell differentiation is confirmed by localization of alpha-actin (original magnification ×300). Note the opaquely stained thick strands, which represent alpha-actin smooth muscle fibers (arrow).*

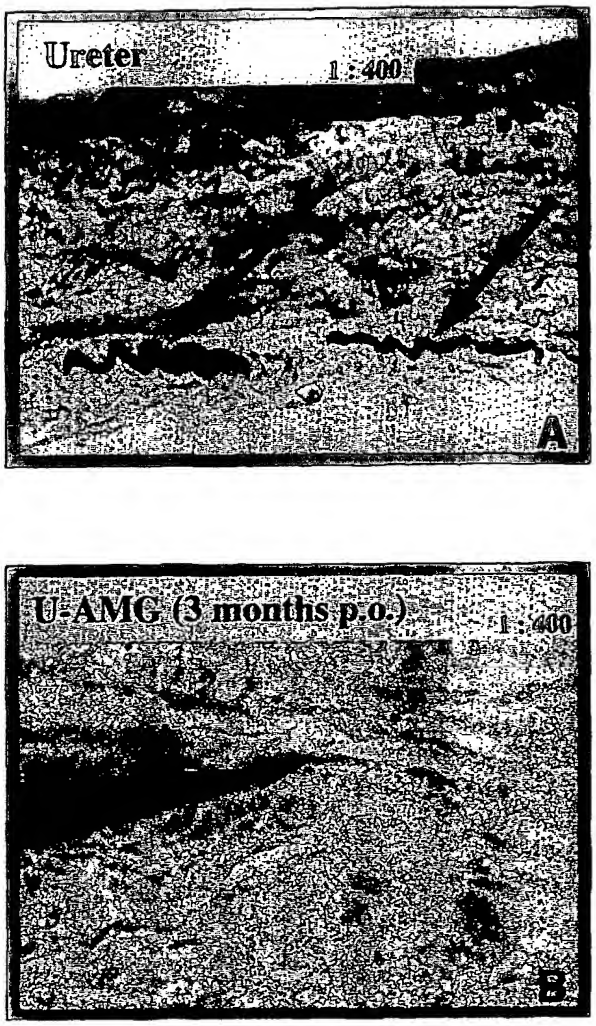


FIGURE 7. *PGP 9.5 staining of normal ureter (A) and graft 3 months postoperatively (B). Nerve regeneration is confirmed (original magnification ×400). Note the lower density of PGP 9.5-positive nerve staining in the graft (arrow) (U-AMG, ureteral acellular matrix graft).*

smooth muscle cells were arranged in parallel rows in the longitudinal direction. The thickness of these muscle bundles seemed to decrease in the central part of the graft. There was no sign of degenerative change such as calcification or necrosis of the smooth muscle layer. The urothelial lining, differentiated muscularization, and surrounding fibrous adventitia appeared qualitatively similar to normal ureteral wall components (see below). In contrast, we noted that the number of nerve fibers was less than in the normal ureter.

Electron Microscopy

Smooth muscle regeneration in the grafted ureteral matrix was confirmed by transmission electron microscopy (Fig. 8). At 3 months, specimens demonstrated a lower density of myofilaments in the graft than in the normal rat ureter. At 4 months, the number of myofilaments was significantly increased. These observations corresponded to the light microscopic findings at the same time. Nerve regeneration was confirmed in 4-month specimens studied by electron microscopy (Fig. 9). Although the number of nerve fibers in the matrix graft was notably less than in the normal ureter, their morphologic characteristics were similar.

COMMENT

Ureteral replacement has long been a challenge for urologists. In other specialties, the success of acellular matrix grafts for organ substitution has been reported (eg, replacement of heart valves, coronary artery bypass, and, in particular, skin).[11–14] Although the concept of free grafts to treat ureteral diseases is not new, none of the materials previously reported has been able to satisfy all the criteria for the ideal substitute (Table I).[15–24] In the present study, complete epithelialization, angiogenesis, and regeneration of smooth muscle fibers and nerves were observed with no signs of rejection.

The reasons for the improved acceptance of the ureteral acellular matrix graft over that of other free ureteral transplants are still unknown. In accordance with our previous experience, we used extremely fine nonabsorbable suture material to minimize immediate postoperative inflammatory reactions, which can result in incrustation or stone formation. Rapid epithelialization, progressive

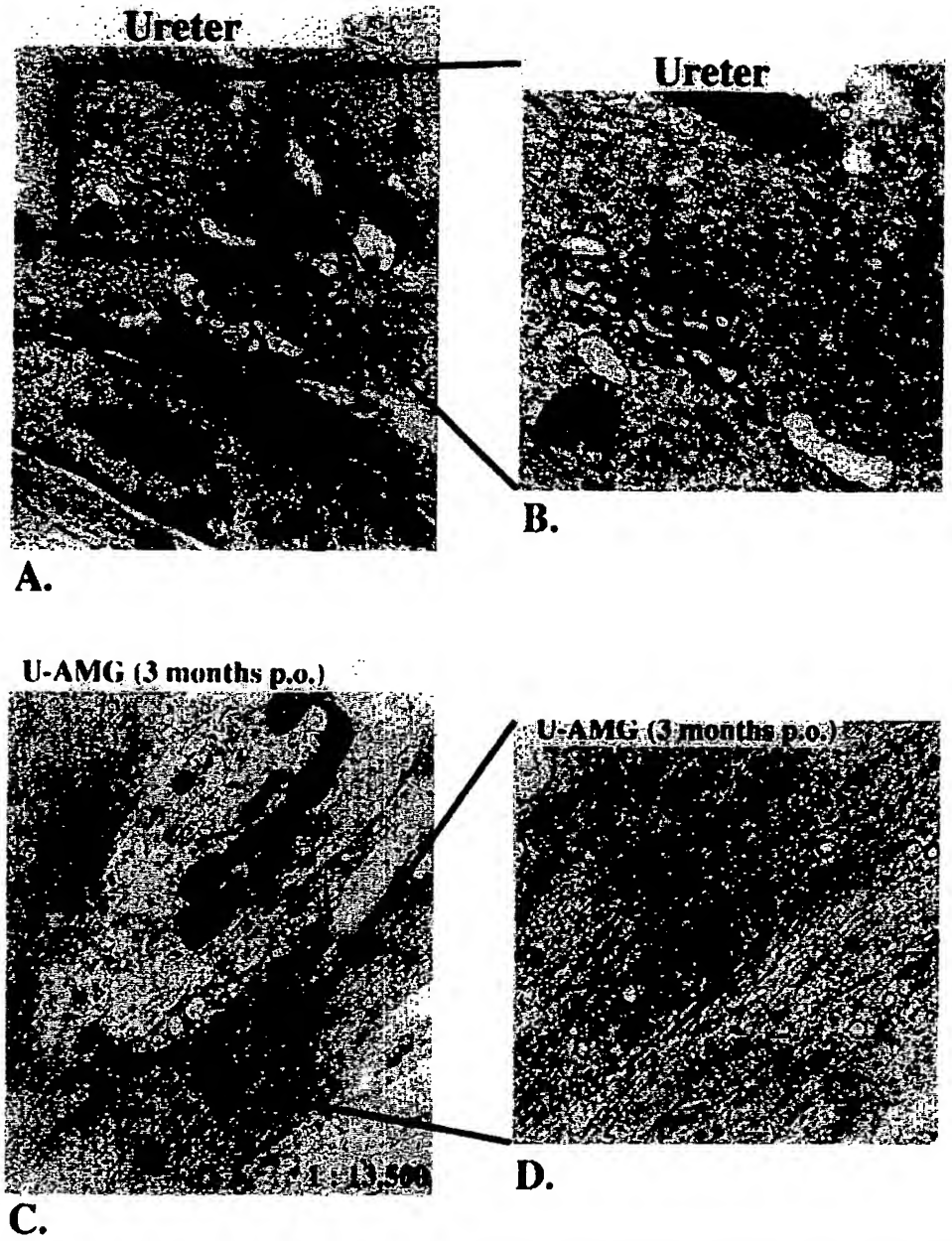
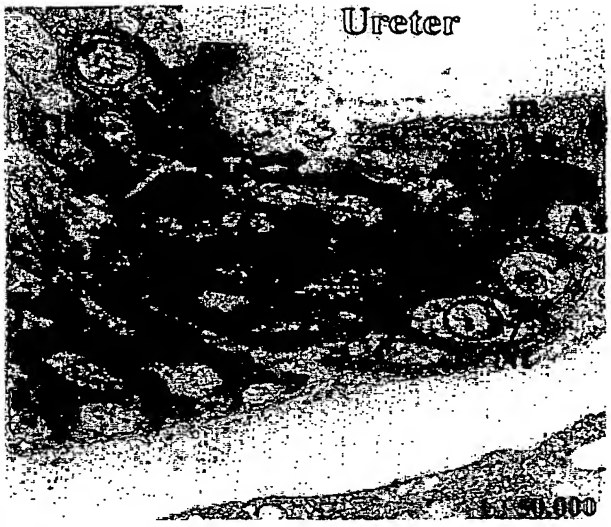


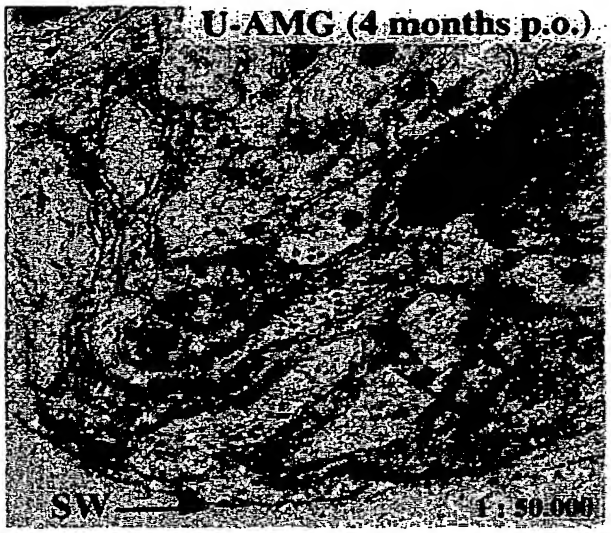
FIGURE 8. *Transmission electron microscopy of normal ureter (A and B) and graft 3 months postoperatively (C and D) (original magnification ×13,500). Note lower density of myofilaments (M) in the graft-regenerated rat ureter than in the normal ureter. Mitochondria (m) are also seen (U-AMG, ureteral acellular matrix graft).*

neoangiogenesis, and continuous smooth muscle regeneration, apparently arising from the adjacent edges of the host ureter, are possible factors responsible. Expression of growth factors seems to play a key role in the mechanisms controlling epithelialization.[25] In our research, studies of transforming growth factor (TGF) expression in the rat bladder acellular matrix graft showed variable induction of TGFα, TGFβ_1, TGFβ_2, and TGFβ_3 transcription, with prominent mRNA expression of TGFα and TGFβ_1 (preliminary results).

In time, early smooth muscle cells matured into normal-appearing smooth muscle bundles. Because smooth muscle production has been reported from pericytes after capillary neovascularization,[26] we hypothesize on the basis of our studies that the source of smooth muscle fibers in the acellular matrix graft is the adjacent edges of the host ureter. Regulating mechanisms of smooth muscle differentation are poorly understood. Besides growth factor expression (as just mentioned), epithelial-mesenchymal interactions are thought to be important for smooth muscle regeneration. In 1961, Taderera[27] demonstrated that the absence of lung epithelium causes failure of both smooth muscle and cartilage differentation. Consistent with these results, Baskin *et al.*[28] reported in 1996 that intact bladder as well as isolated blad-



A.



B.

FIGURE 9. *Transmission electron microscopy of normal ureter (A) and graft 4 months postoperatively (B) (original magnification ×50,000). The graft-regenerated rat ureter shows morphologically intact nerves with axons (Ax), mitochondria (m), neurotubules (Nt), neurofilaments (Nf), and Schwann cell sheath (SW) (U-AMG, ureteral acellular matrix graft).*

der mesenchyme recombined with bladder urothelium from rat fetuses, when grafted under the renal capsule of adult rats, demonstrated expression of smooth muscle differentiation; however, grafts of bladder mesenchyme alone failed to induce smooth muscle differentiation. Therefore, early epithelialization in the graft may be highly important for smooth muscle differentation.

The observation that the overall (PGP 9.5-positive) density of the reinnervation of the ureteral acellular matrix graft was significantly lower than that of the normal ureter may indicate that cell-derived factors are needed to achieve normal levels of innervation. A similar experience has been described by Gavazzi *et al.*[29] They showed that, after grafting of frozen and thawed acellular cerebral blood vessels in oculo, the reinnervation of the graft was less than that of the control, and PGP 9.5-positive nerves appeared less dense on the transplants as well. However, in the present study regeneration of nerves was confirmed by light and electron microscopy and may increase over a longer period of time to achieve functional capacities.

TABLE I. *Criteria for the ideal ureteral substitute

Close histologic resemblance to normal ureter
Peristaltic activity synchronous with host ureter
Adequate blood supply
Free transport of urine
Impermeable, nonabsorptive lining
No immunologic reaction
No stone formation
Technical ease
No stricture formation at the anastomotic site
Normal innervation and adequate pharmacologic response

* *Summarized from Baum* et al.[2]

In conclusion, the ureteral acellular matrix graft appears to promote the regeneration of all ureteral wall components. These results may indicate that the graft has a potential for functional neomuscularization that will result in its maintenance as a physiologic ureteral wall. Tube migration with consequent obstruction at the level of the stent, causing hydroureteronephrosis, was not avoidable. It would thus be reasonable to undertake further study in a larger animal model. Detailed functional and molecular biologic experiments are needed to evaluate whether the ureteral acellular matrix graft as a ureteral substitute can conduct peristaltic activity in coordination with the host components to preserve the functional integrity of the renal parenchyma. The findings of regenerated ureteral wall components are encouraging and support the clinical potential of the acellular matrix graft in genitourinary tract reconstruction.

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HOMOLOGOUS ACELLULAR MATRIX GRAFT FOR URETHRAL RECONSTRUCTION IN THE RABBIT: HISTOLOGICAL AND FUNCTIONAL EVALUATION

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ABSTRACT

Objective: To evaluate urethral replacement by a free homologous graft of acellular urethral matrix in a rabbit model.

Materials and Methods: In 30 male New Zealand rabbits, a 0.8 to 1.1 cm. segment of the urethra was resected, replaced with an acellular matrix graft of 1.0 to 1.5 cm. (mean 1.3 cm.), and placed on an 8F feeding tube. Additionally 4 animals underwent sham operation. At varying intervals before sacrifice (from 10 days to 8 months), the animals underwent urodynamic evaluation and retrograde urethrography (for which 4 untreated rabbits served as control). The grafted specimens were prepared for evaluation histologically and by reverse-transcription polymerase chain reaction (RT-PCR).

Results: In all animals, the acellular matrix graft remained in its original position. Histological examination showed complete epithelialization and progressive vessel infiltration. At 3 months, smooth muscle bundles were first observed infiltrating the matrix at the end-to-end anastomosis; after 6 months, the smooth muscle bundles had grown into one-third of the matrix. Urodynamics did not detect any difference between the control and matrix-grafted animals in bladder volume, leak-point pressure and residual volume. RT-PCR detected an increase in IGF mRNA in the graft between week 3 and month 6 and in HB-EGF mRNA after day 10 through month 3. TGF-α mRNA was not detected; TGF-β mRNA was unchanged from normal urethral tissue. By 8 months, the host and implant could not be differentiated by urethrography.

Conclusion: The acellular urethral matrix allows single-stage urethral reconstruction. All tissue components were seen in the grafted matrix after 3 months, with further improvement over time; however, the smooth muscle in the matrix was less than in normal rabbit urethra and was not well oriented. RT-PCR revealed the importance of time-dependent growth factor influences during regeneration.

KEY WORDS: urethra; transplantation, homologous; graft survival, rabbit model; urodynamics; growth factors

The urologist remains challenged by urethral defects and injuries. Indeed, the variety of surgical techniques and the search for the best material attest to this problem. Even with high success rates (good cosmetic results and function), no method guarantees freedom from complications such as fistula and stenosis. The search for alternative surgical treatment to avoid multistage surgeries (time or location) suggests that a biodegradable material may provide more consistent results. The tissue properties should be similar to the original. In previous work, an acellular scaffold of the bladder and the ureter was used for organ-specific regeneration, with demonstrably good results histologically and functionally.[1,2] In this study, we evaluate the homologous acellular matrix used to bridge a urethral gap in the rabbit.

MATERIALS AND METHODS

Male New Zealand rabbits (n = 38), weight 3.0 to 3.5 kg., were caged individually (16C constant temperature, 47% humidity, 12-hour light/dark cycle) with free access to standard laboratory chow and tap water. Beginning four days before surgery, the animals wore a modified Elizabethan collar for 10 hours per day, then continuously after surgery until urethral stent removal at postoperative day 7.

The matrix graft was prepared as described previously,[1,2] with the following modifications, according to a method adapted from Meezan et al.[3] A polyethylene 10-F Argyle feeding tube (Sherwood Medical; St. Louis, MO) was inserted through the excised New Zealand rabbit urethra (obtained from our institution's tissue-sharing program). In contradistinction to our previous studies, the urethra was not inverted nor was the mucosa scraped off. The tissue was placed in 10 mM phosphate-buffered saline (PBS, pH 7.0) and 1% sodium azide for partial cell lysis. Cell lysis was completed in 1 M sodium chloride containing 200 Kunitz units DNase (Sigma; St. Louis, MO). The specimens were treated in 50 ml. of 4% sodium desoxycholate containing 0.1% sodium azide and stirred for 5 to 6 hours to solubilize the lipid membranes. This step was repeated once. The acellular matrix was washed three times in PBS and stored in 10% neomycin sulfate at 4C until grafted.

Anesthesia was induced with ketamine (40 mg./kg. i.m.) and midazolam (2 mg./kg. i.m.) and was maintained after endotracheal intubation with isofluorane (3%) inhalation. The rabbits were mechanically ventilated with 100% oxygen (2 L/min.) via the endotracheal tube. Arterial blood pressure, pulse and respiration rate, body temperature and EKG were monitored continuously throughout the surgical procedure.

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An ear vein was cannulated for continuous dextrose 2.5% (40 ml./hr.) infusion. A warm-water mattress maintained body temperature.

Surgical Procedure. After the urethra was catheterized by an 8F Argyle® feeding tube, the pendulous urethra was exposed through a ventral midline, penile skin incision (1.0 to 1.3 cm.) and mobilized from the corpora cavernosa. The graft and sham operations were performed with the aid of microsurgical loupes (25X magnification).

Partial urethral replacement by acellular matrix. A 0.8 to 1.1 cm. segment of the urethra was excised and replaced by the homologous acellular matrix graft (1.0 to 1.5 cm., mean 1.3 cm.) in 30 animals divided in 6 subgroups (for sacrifice at 10 days, 3 or 6 weeks, 3, 6 or 8 months, 5 per group). The graft was sutured in place by interrupted 7–0 polyglactin sutures (Vicryl, Ethicon Inc., Somerville, NJ), bridging the urethral defect end-to-end (fig. 1). Monofilament 7–0 nonabsorbable Dermalon sutures were used on three points (9, 12 and 3 o'clock) for matrix border identification.

Sham operation. A segment of the exposed urethra was transected in two places (1.3 to 1.5 cm. apart) and reanastomosed as described above in 4 rabbits (for sacrifice at 3 and 6 months, 2 per group).

To promote healing, the wound was closed in layers with running 7–0 polyglactin suture, and the skin with an inverted running 4–0 polyglactin suture (Vicryl Rapid, Ethicon). The feeding tube was fixed to the meatus with 2–0 silk suture and shortened so that only 0.5 cm. was visible.

The rabbits received enrofloxacin (Baytril, Bayer Corp., Shawnee, KA) intraoperatively (11.35 mg. i.v.) and twice a day orally until stent removal at day 7.

Radiologic evaluation. Both groups underwent retrograde urethrography in preanesthesia to confirm normal anatomy. In addition, 4 animals acted as control for the functional evaluations. A modified 8-F feeding tube was inserted into the meatus and fixed to the penile skin with a 2-zero silk suture. Urethrography was performed after injection of 1.5 ml. diatrizoate meglumine 60%; x-ray parameters were 2.5 mA and 56 kV by a mobile x-ray system (General Electric AMX 3 model No. 46-217900G2, Milwaukee, WI). Before sacrifice, all animals (n = 38 [30 matrix, 4 sham, 4 control]) underwent retrograde urethrography in the supine position. Long-term animals (8 months) had two further followup evaluations at 3 and 6 months.

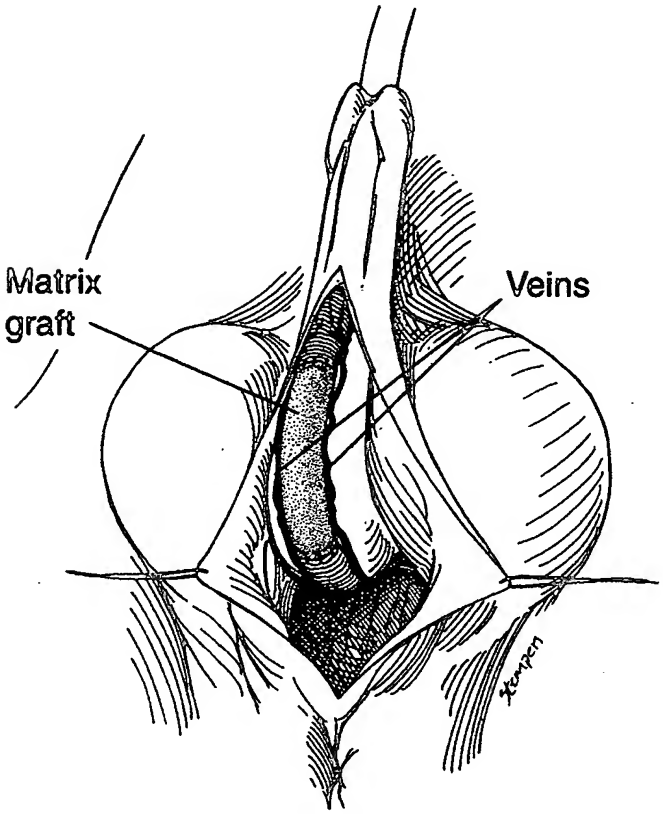

FIG. 1. Artist's rendition of surgical field. Matrix (1.5 cm.) is anastomosed by single sutures, preserving vein.

Urodynamics. After retrograde urethrography, open urodynamic evaluation was performed after suprapubic midline incision and cystostomy by an 8-F Argyle feeding tube connected to a Uniflow transducer (Baxter Healthcare Corp., Deerfield, IL) via a three-way stopcock. The pressures were recorded continuously and evaluated by Labview 4.0 (National Instruments, Austin, TX) through a Macintosh Quadra 800 computer, according to a protocol modified from Celayir et al.[4] After an equilibration period of 15 minutes, each rabbit underwent at least three consecutive cystometric measurements. The bladder was filled by a 1711 Uropump (LifeTech, Inc.) with 0.9% sodium chloride at 4 ml./min. The evaluation included a urethral pressure profile, which was repeated twice for each animal.

Light microscopy. Specimens were fixed in 10% buffered formalin for at least 24 hours. After dehydration in graded ethanol solutions, the specimens were embedded in paraffin, sectioned (4 μm.), and stained with hematoxylin and eosin (H & E), trichrome, and immunostained for α-actin. Paraffin sections were hydrated to water. After blocking endogenous peroxidase (20 minutes with 0.3% H_2O_2 in methanol) and nonspecific protein binding (overnight with 3% normal horse serum in PBS, pH 7.4, containing 0.3% Triton X-100), sections were incubated for 60 minutes at room temperature with mouse monoclonal anti-smooth-muscle α-actin (1:6000 in PBS; Sigma Chemicals, St. Louis, MO). After washing with buffer, sections were immunostained with the avidin-biotin-peroxidase method (Elite ABC, Vector Laboratories; Burlingame, CA), with diaminobenzidine plus hydrogen peroxide as the chromagen. Sections were counterstained with hematoxylin. As a negative control, sections were immunostained without exposure to primary antibody.[5]

Pure graft specimens were also prepared for light microscopy (H & E and trichrome) and electron microscopy to confirm the structure as an acellular scaffold with collagen and elastin fibers, demonstrating the effectiveness of the matrix preparation process.

The number and diameter of vessels in the area of the anastomosis and in the middle of the graft, as well as in normal urethral tissue, were counted and measured in a 5 × 5 field (magnification 40x, α-actin-stained slides). The mean of four random fields was calculated.

RNA preparation. At sacrifice, 10 days, 3 and 6 weeks, and 3, 6 and 8 months postoperatively, freshly dissected matrix tissue from each group was homogenized in Tri-Reagent RNA/DNA/Protein isolation reagent (Molecular Research Center; Cincinnati, OH). According to the supplier's recommended procedure, RNA was treated with DNase I to remove contaminating DNA traces. RNA integrity was examined by agarose gel electrophoresis.

RT-PCR analysis. The cellular mRNA was reverse-transcribed with SuperScriptase reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) and its accompanying reagents into a "library" of complementary DNA (cDNA) and used to analyze the expression patterns of various genes in the PCR step. The PCR mixture consisted of 10 ng. each of a primer pair and reagents supplied with the Taq polymerase (Life Technologies, Inc.). PCR was performed in the DNA engine thermocycler (MJ Research, Inc., Watertown, MA) under calculated temperature control. The cycling program was set for 37 cycles of 94C, 5 seconds; 55C, 5 seconds; 72C, 10 seconds; followed by one cycle of 72C, 5 minutes. The PCR product was electrophoresed in a 1.5% agarose gel, visualized

Primers used for RT-PCR

EGF Primers		
EGFs	AGGCAGCATGCTGAAGCCC	
EGFa	CCAGCAAATCCTTTCAAACAC	423 bp
IGF Primers		
IGF1s	TGCTTCCGGAGCTGTGATCT	human 528 bp
IGF1a	CAGAGAGGAATTTAGTGCAAC	rat 579 bp
TGF Primers		
TGF-b1s	CGGCAGCTGTACATTGACTT	
TGF-b1a	TCAGCTGCACTTGCAGGAGC	278 bp
Actin Primers		
actin-s	TCTACAATGAGCTGCGTGTG	
actin-a	ATCTCCTTCTGCATCCTGTC	682 bp

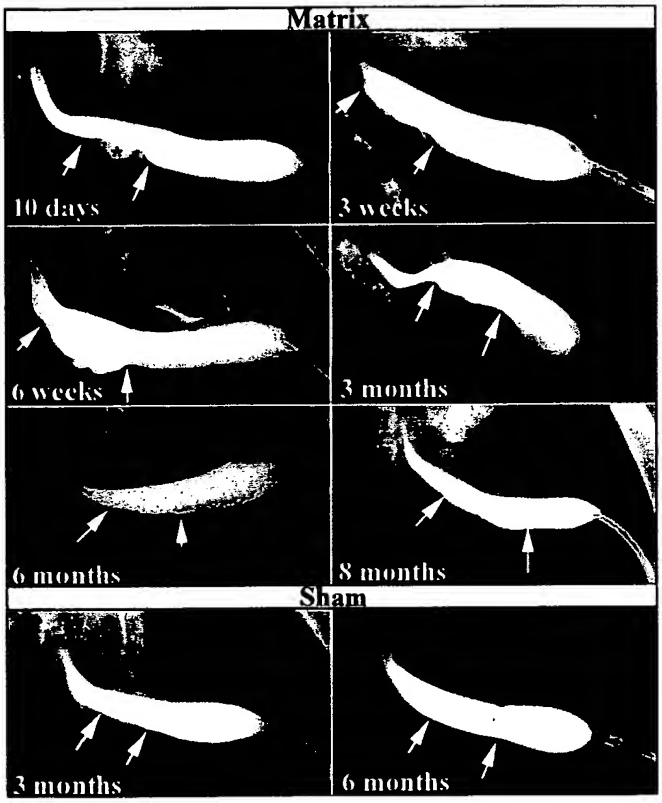

FIG. 2. Postoperative retrograde urethrography in matrix-grafted and sham-operated animals. After period of rigidity and permeability (day 10), graft becomes hyperflexible (week 6). At 8 months, both groups have similar appearance.

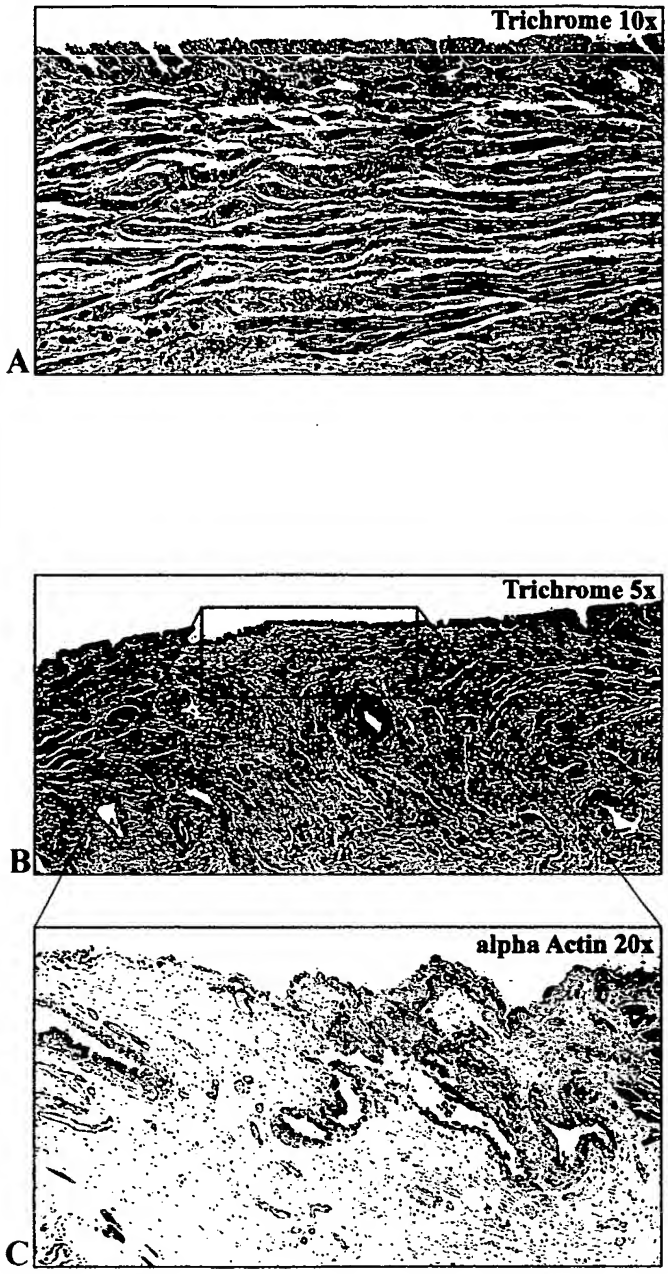

FIG. 3. Light microscopy: *A*, normal rabbit urethra (trichrome, ×10 magnification) reveals urothelium and well oriented smooth muscle cells. *B*, at site of transection in sham-operated animals (6 months) (trichrome, 5x), higher magnification (*C*) shows no smooth muscle cells on α-actin staining (20x).

by UV fluorescence, and recorded by a digital camera connected to a computer. Densitometry was performed to determine the relative levels of gene expression, with the TGF-β1 gene PCR result as a reference.

Design of oligonucleotide primers. For RT-PCR analysis of mRNA expression of various genes (table) and for DNA sequence analysis of the RT-PCR products, several pairs of oligonucleotide primers have been designed and synthesized. To avoid the generation of PCR products from residual contaminating genomic DNA, the two primers in each primer pair are selected from two separate exons whenever the gene structure information is available. The MacVector computer program (Oxford, Inc.) was used to search the GenBank database and analyze the retrieved gene sequences.

RESULTS

No animal died during the study. A fistula at the 12 o'clock position was observed in 4 of 24 matrix-implanted animals (1 at 3 weeks, 2 at 6 weeks, and 1 at 3 months). Light microscopy showed these to have been caused by the nonabsorbable Dermalon suture used for marking.

Retrograde urethrography in the matrix-implanted animals showed (fig. 2) that at 10 days extravasation was demonstrable in the middle of the implant. The matrix was minimally expanded at the area of anastomosis only. At 3 weeks, minimal extravasation was detected in the matrix implant, and flexibility was increased. At 6 weeks, the neourethra had become hyperflexible. Extravasation was no longer seen. At 3 months, the hyperflexibility had decreased and the diameter of the implant was similar to the host's. There was no sign of stricture. At 6 months, no difference could be discerned between implant and host and the borders were difficult to differentiate. At 8 months, the matrix had adapted further to the host. In the sham-operated animals, no difference from normal urethra at 3 or 6 months was demonstrable.

Urodynamic evaluation showed no noteworthy differences

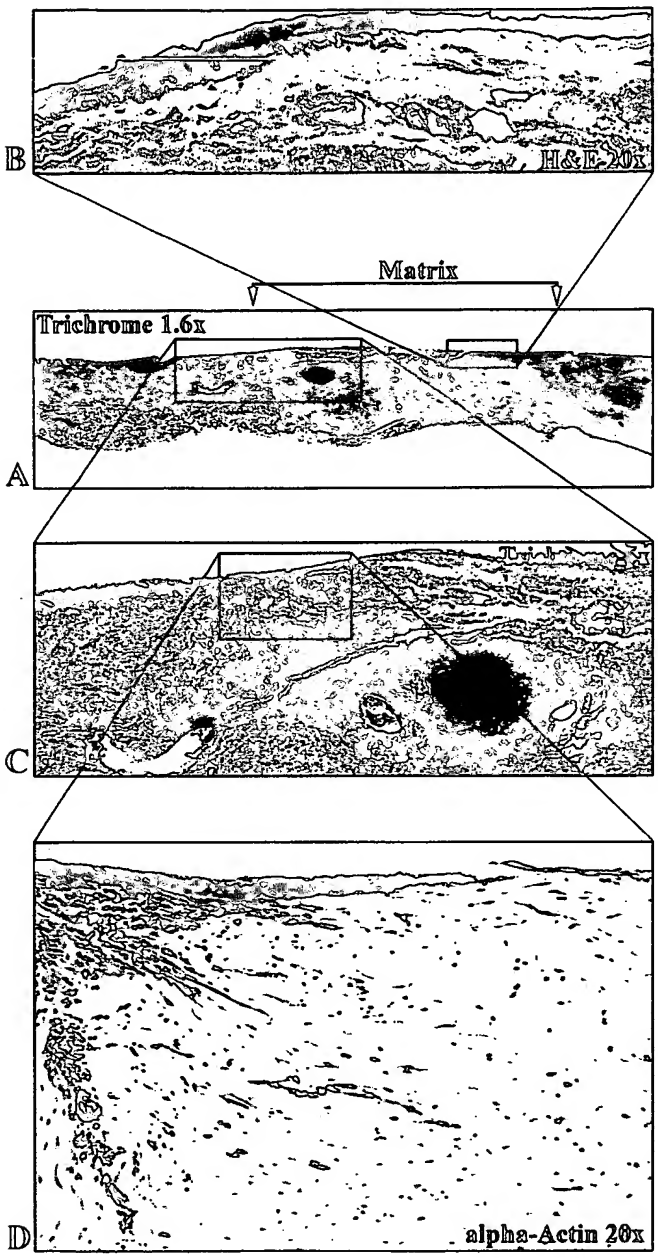

FIG. 4. Light microscopy, matrix-grafted animals, day 10: *A*, longitudinal section (trichrome, 1.6x). *B*, at higher magnification (H & E, ×20), epithelialization of graft borders is seen, with no epithelialization in middle of the matrix. *C*, in another detail of *A*, anastomosis between host and graft is highlighted (trichrome, 5x). *D*, in this higher magnification of *C*, submucosal infiltration of myofibroblasts into the gap between the host and the graft tissue is apparent (α-actin, ×20).

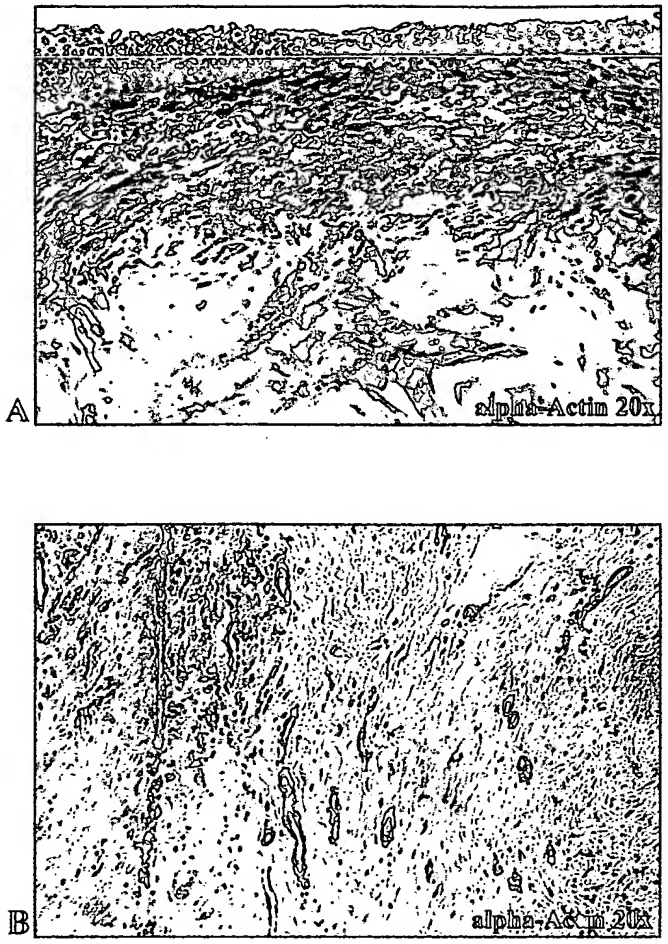

FIG. 5. Light microscopy, week 3 (α-actin, ×20): *A*, matrix lumen is covered by irregular epithelium, and submucosa is filled with myofibroblasts infiltrating the matrix. *B*, deep into central matrix, vessels outlined by smooth muscle cells grow into scaffold.

in bladder capacity, leak-point pressure, residual volume and urethral pressure profile among the untreated, sham-operated or matrix-implanted animals.

Light and electron microscopy of the pure matrix confirmed the effectiveness of the matrix preparation process. The acellular graft appeared as an intact framework consisting of elastin and collagen fibers. Electron microscopy demonstrated the intact urethral matrix surface and the scaffold structure with no cellular elements.

Macroscopically, adhesions were noted in both groups (matrix-implanted and sham-operated) and were dependent on postoperative time.

On light microscopy, the loose collagen and elastin fiber composition of the normal rabbit urethra consists of numerous outer longitudinally oriented smooth muscle bundles and inner nutritional vessels. The urethral lumen is lined with prismatic and cuboidal epithelium (fig. 3, *A*). In contrast, in the sham-operated animals the reanastomosed transected areas showed no smooth muscle ingrowth after 6 weeks and 3 and 6 months; the area was filled by collagen. The urethra and corpus spongiosum between these cuts were normal (fig. 3, *B* and *C*), and fine vessels bridged the healthy tissue.

The matrix-implanted specimens were examined at six time points: At 10 days, epithelial cells had migrated from each host side, covering half of the matrix with a single epithelial layer (fig. 4, *A-C*). At this time small vessels were developing in the matrix. The connective tissue between host and matrix showed high α-actin staining, representing myofibroblasts. Myofibroblasts and vessels followed the matrix scaffold (fig. 4, *D*). The matrix (especially around the resorbable sutures) and adjacent areas of the host tissue were infiltrated with inflammatory cells (fig. 4, *D*).

At 3 weeks, the urethral lumen was completely covered by epithelial cells, although still irregularly in some areas (fig. 5, *A*). Vessels of a larger diameter than those seen at 10 days infiltrated the complete matrix. In addition to the positive α-actin staining in the smooth muscle cells of the vessels, the submucosa showed high expression of α-actin in myofibroblasts (fig. 5, *B*). The matrix scaffold appeared less dense than at day 10. The sutures were almost resorbed.

At 6 weeks, the epithelium was regular and equivalent to the normal urethra (fig. 6, *A*). Vessels ran along the basal membrane, and in the remaining matrix their diameter was

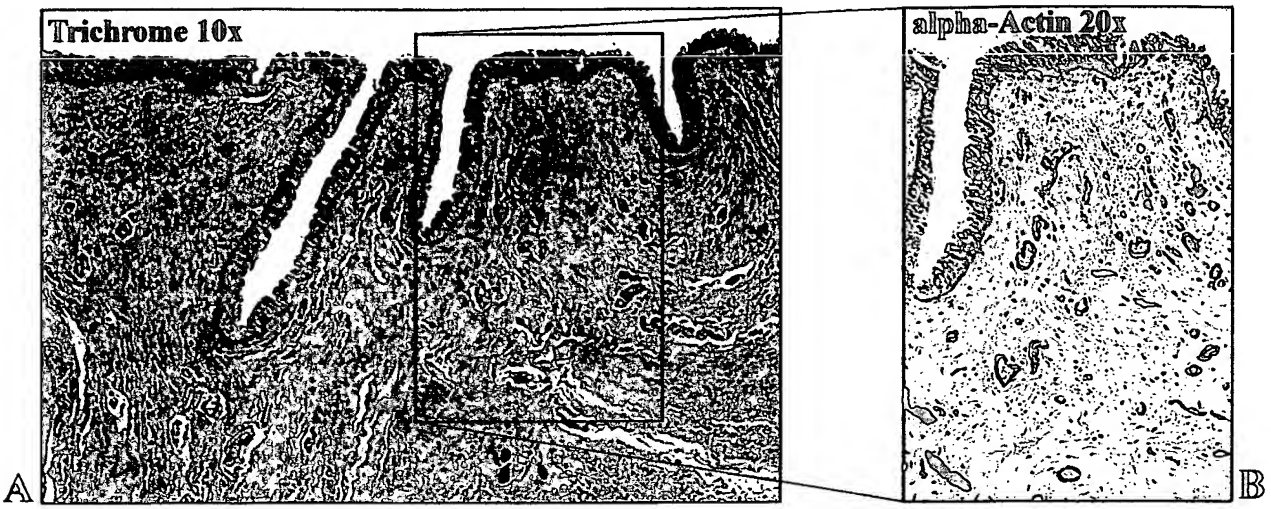

FIG. 6. Light microscopy, week 6: *A*, regular epithelium covers entire graft (trichrome, ×10). *B*, at higher magnification (α-actin ×20), the good vessel supply in submucosa is apparent. In lower left corner, first smooth muscle cells are seen. Myofibroblasts have completely disappeared.

further increased. Alpha-actin staining was seen only in the vessel walls. The myofibroblasts and the last white blood cells had disappeared (fig. 6, *B*).

At 3 months, the regular prismatic and cuboidal epithelium had begun to exhibit folds (fig. 7, *A* and *B*). The connective tissue of the submucosa was loose, as in the normal urethra, in contrast to the transected areas of the sham-operated animals. The vessel number and diameter were greater than those in the sham-operated rabbits (fig. 7, *C*). In some specimens smooth muscle cells had begun to infiltrate the matrix at the end-to-end anastomosis, and the overlying tissue contained some smooth muscle bundles (fig. 7, *D*). Trichrome staining demonstrated the entire matrix scaffold to be less compact (fig. 7, *A*).

At 6 months, longitudinal smooth-muscle-cell bundles were seen in one-third of the matrix, less well-oriented than in the normal urethra (fig. 8, *A* and *B*). In α-actin-stained sections, irregular areas with few muscle cells were seen, except in the matrix center (fig. 8, *C*). However, additional cross-sections demonstrated a significant increase of longitudinal vessels.

At 8 months, longitudinal sections showed only minor changes, with further smooth-muscle-cell ingrowth.

Evaluation of vessel ingrowth into the homologous acellular matrix graft, measured by number and diameter in the area of anastomosis and in the middle of the graft over the time (0 days to 8 months), demonstrated an initial increase in number followed by a decrease that was simultaneous with an increase in vessel diameter (fig. 9).

On RT-PCR, mRNA expression was observed at different intervals postoperatively in the acellular matrix graft. Insulin-like growth factor (IGF) mRNA was detected by week 3, increasing to a maximum at 6 weeks and returning to normal after 6 months (fig. 10). Expression of heparin-binding epidermal growth factor (HB-EGF) mRNA increased notably from week 3 to month 6, but HB-EGF mRNA was not detected in normal urethral tissue or in the matrix-implanted samples at 10 days (fig. 10, *B*). Expression of transforming growth factor (TGF)-β1 mRNA was similar in all specimens (fig. 10, *C*). No TGF-α mRNA (fig. 10, *D*) and β-actin expression was detected, as had been seen in the rat model;[6] therefore TGF-β1 mRNA was used as the internal standard for baseline gene expression.

DISCUSSION

The importance of urethral reconstruction in urology is underscored by the many efforts to improve surgical technique and materials. Previous research has shown that biodegradable materials such as hyaluronic acid or hydroxyacetic acid polymers have the best potential for regeneration and function,[7–9] although the degree and quality of regeneration have varied. In the rabbit urethra, regeneration of endothelium and submucosa was observed, but no formation of smooth muscle cells.[9] Encouraged by the results of our bladder regeneration studies with an acellular matrix graft, we expanded our investigation to examine partial urethral replacement. The advantages of this approach are a shorter operative time with only one intervention and minimal reaction. In the present study, complete luminal epithelialization, rapid angiogenesis, and smooth-muscle-cell bundle growth were observed with no signs of rejection. Retrograde urethrography, the clinical gold standard for surgical followup, documented the ongoing regenerative changes and the functional improvement over time.

Neovascularization increased over time. At first, thin vessels were seen extending into the matrix from the host tissue; ultimately, the vessels were seen throughout the matrix in number and diameter similar to those in the host-tissue border. Vascular smooth muscle cells were detected by α-actin stain after 3 weeks. The longitudinal vessels increased in number markedly between months 3 and 6, with no further changes at 8 months. The nutritional vessels of the epithelium were similar to those in the normal urethra by month 3.

At 10 days, only myofibroblasts were stained with α-actin. The increase of myofibroblasts in the anastomosis at day 10 and the accumulation in the submucosa after week 3 are an important step in wound healing.[10] It is not known if myofibroblasts differentiate into smooth muscle cells, although they are very similar in ultrastructure, and recently published results have supported this conjecture.[11,12] Single smooth muscle cells were seen in the submucosa at 6 weeks. These single cells did not bear a close relationship to vessels or even to the host tissue, but were seen in areas where there had been a high myofibroblast accumulation. This is supported by the report of Wu et al in which myofibroblasts infiltrated and subsequently differentiated into smooth muscle cells,[13] although the basis for this differentiation is not known.

Over the 8 months of observation, the ingrowth of smooth muscle cells and bundles was not continuous. Improvement was most demonstrable between 3 and 6 months. Smooth-muscle-cell bundles were observed growing into one-third of the matrix scaffold, not only longitudinally but also with a

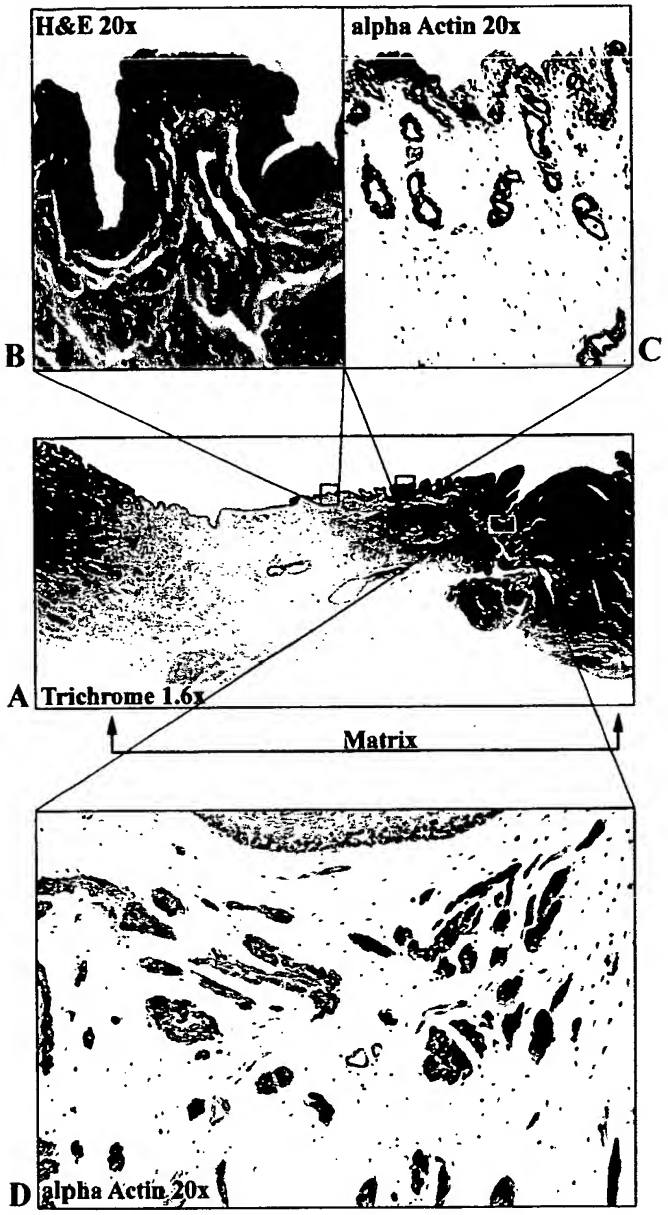

FIG. 7. Light microscopy, month 3: *A*, longitudinal section of the entire graft with host tissue at each side (trichrome, 1.6x). *B* and *C* document regular epithelium (H & E ×20) and increased number and diameter of blood vessels (α-actin, ×20). *D*, in this detail, first smooth muscle bundles are demonstrable (α-actin ×20).

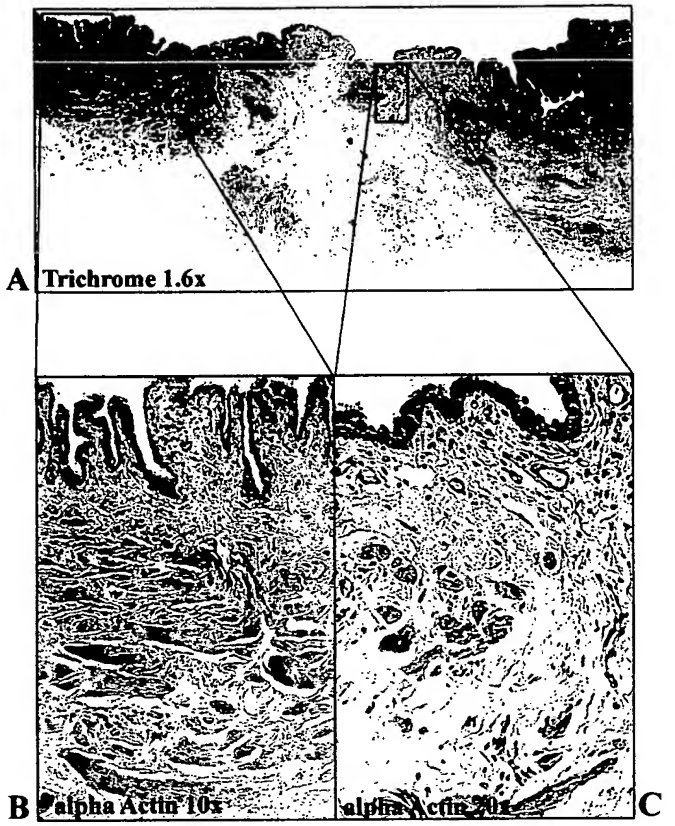

FIG. 8. Light microscopy, month 6: *A*, in this longitudinal view (trichrome, 1.6x) with adjoining host tissue at left, smooth muscle bundles (*B*) are seen migrating into about one-third of the matrix scaffold (α-actin, ×10). Compared with normal urethral tissue, the muscle bundles are not well organized longitudinally. *C*, graft center documents first smooth muscle fibers (α-actin, ×20) with single smooth muscle cells irregularly sited over central third of remaining graft.

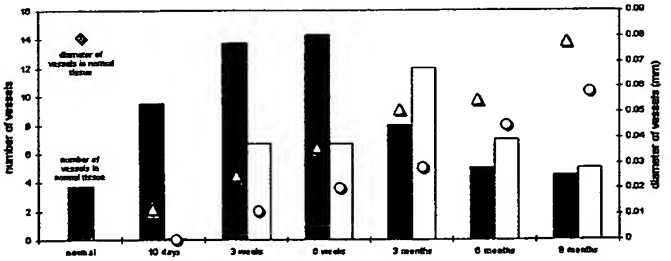

FIG. 9. Vessel development in acellular matrix graft in comparison with that in normal urethra. Number of vessels: black bar = area of anastomosis; white bar = middle of graft. Diameter of vessels: open triangle = area of anastomosis; open circle = middle of graft.

thicker submucosa. Several cells over the midsection of the formerly acellular matrix were seen on α-actin staining, although not trichrome, and some bundles without apparent orientation were seen in the center of the matrix.

To understand the changes through which the acellular matrix goes to become a functional organ part, a review of growth-factor function is helpful. Dahms et al have reported TGF-α mRNA and TGF-β1 mRNA upregulation in the rat model bladder acellular matrix graft, and TGF-α has been observed in urine after bladder manipulation and augmentation.[6] However, TGF-α mRNA was not detected in our grafted samples. That this and β-actin mRNA were not detected might owe to the lack of commercially available rabbit primers. TGF-β1 mRNA was detected at the same levels in all samples, both normal urethra and matrix tissue, and TGF-β1 has been characterized as causing smooth muscle cells to be more adherent[14] and certain smooth-muscle-cell types to inhibit growth.[15] It is unknown why it increased significantly over 6 months in the rat model bladder augmentation study but not in the present study, although this difference may be species-dependent. The small amount detected has been characterized as latent TGF-β1.[16] IGF increased markedly in the graft tissue after 10 days when compared with the normal urethral tissue and decreased to normal amounts by month 6. An overexpression of IGF stimulates smooth-muscle-cell hyperplasia;[17] increased IGF may be a factor in smooth-muscle-cell ingrowth in the matrix. HB-EGF is not expressed in the normal rabbit urethra, but significant levels were detected after day 10 in the grafted tissue. HB-EGF in vivo has been described to promote neovascularization in the rabbit cornea, to induce the migration of bovine endothelial cells, and to release endothelial cell mitogenic activity from bovine vascular smooth muscle

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HETEROLOGOUS ACELLULAR MATRIX GRAFT FOR RECONSTRUCTION OF THE RABBIT URETHRA: HISTOLOGICAL AND FUNCTIONAL EVALUATION

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ABSTRACT

Purpose: In a rabbit model we evaluated urethral replacement by a free heterologous dog acellular matrix graft and compared these results with those of a homologous graft with the exclusion of antigenicity as a major goal.

Materials and Methods: In 14 male New Zealand rabbits a 0.8 to 1.1 cm. segment of urethra was resected and replaced with a tubular acellular 1.0 to 1.5 cm. (mean 1.3) urethral matrix graft placed on an 8Fr feeding tube. Seven animals received a rabbit graft, 7 received a canine graft and 3 untreated rabbits served as controls. All animals underwent urethral pressure profile determination and retrograde urethrography before 8 and 6 were sacrificed at 6 and 8 months, respectively. Grafted and normal specimens were evaluated by histological testing.

Results: In all animals the acellular matrix graft remained in its original position. Histological examination showed complete epithelialization and progressive vessel infiltration. At 6 months more than a third of the homologous grafts had smooth muscle bundles but the heterologous grafts had only poorly disseminated smooth muscle. Picrosirius red stain demonstrated a shift in the ratio of collagen types I-to-III with an increase in type III in the processed homologous and heterologous matrices that did not change significantly postoperatively. At 8 months the urethral pressure profile detected no difference in control and matrix grafted animals, and urethrography did not readily differentiate host from implant.

Conclusions: In the heterologous matrix all tissue components were present after 6 months with no signs of rejection and even gradual improvement with time. However, regenerated smooth muscle did not equal that in normal rabbit urethra and it was not well oriented. Even after 8 months only a few disseminated smooth muscle cells were evident. Most α-actin positive cells were surrounding the vessels. Although function was normal, the alteration in the collagen ratio effected by matrix production indicated that the matrix collagen appeared not to have been replaced by host collagen. The increase in collagen type III may explain the lack of stricture in the grafted animals on normal retrourethrography.

KEY WORDS: urethra; rabbits; graft survival; muscle, smooth; collagen

Urethral reconstruction continues to challenge the urologist. In previous studies from our laboratory acellular matrices of the bladder[1–3] and ureter[4] as well as a homologous urethral matrix[5] were used for organ specific regeneration with good histological and functional results. For successful clinical application acceptance of the graft material is essential. In our previous studies of the bladder we tested 4 species after successfully grafting homologous bladder acellular matrix and found that they were all well accepted by the recipient. In our current study we determined whether the same observation was true for urethral reconstruction, that is whether heterologous grafts may be used without inducing antigenicity. Acellular matrix obtained from the dog urethra was grafted onto a rabbit urethra and compared with a homologous graft.

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MATERIALS AND METHODS

Animals and graft. A total of 14 male New Zealand rabbits weighing 3.0 to 3.5 kg. were caged individually at a constant temperature of 16C with 47% humidity and a 12-hour light-dark cycle with free access to standard laboratory chow and tap water. Beginning 4 days before surgery the animals wore a modified Elizabethan collar for 10 hours daily and then continuously after surgery until urethral stent removal at postoperative day 7.

The matrix graft was prepared according to a previously reported method.[5] A polyethylene 10Fr Argyle feeding tube (Sherwood Medical, St. Louis, Missouri) was inserted through the excised rabbit or dog urethra obtained from the tissue sharing program at our institution. The tissue was placed in 10 mM. phosphate buffered saline (PBS), pH 7.0, and 1% sodium azide for partial cell lysis. Cell lysis was completed in 1 M. sodium chloride containing 200 Kunitz units deoxyribonuclease (Sigma Chemical Co., St. Louis, Missouri) and stirred for 24 to 72 hours. Specimens were treated in 50 ml. 4% sodium desoxycholate containing 0.1% sodium azide and stirred for 24 hours to solubilize the lipid membranes. This step was repeated once. The acellular matrix

tube was washed 3 times in PBS and stored in 10% neomycin sulfate at 4C until grafted (fig. 1).

Anesthesia was induced with 40 mg./kg. ketamine and 2 mg./kg. midazolam given intramuscularly and maintained after endotracheal intubation with 3% isofluorane inhalation. The rabbits were mechanically ventilated with 100% oxygen at a rate of 2 l. per minute via the endotracheal tube. Arterial blood pressure, pulse and respiration rate, body temperature and electrocardiography were monitored continuously throughout the surgical procedure. An ear vein was cannulated for continuous 2.5% dextrose infusion at a rate of 40 ml. per hour. A warm water mattress maintained body temperature.

Surgical procedure. After the urethra was catheterized by an 8Fr Argyle feeding tube the pendulous urethra was exposed through a 1.0 to 1.3 cm. ventral midline penile skin incision and mobilized from the corpora cavernosa. Surgery was performed with the aid of microsurgical loupes at ×25 magnification.

Animals were divided into 2 groups of 7 each for homologous and heterologous grafts, and a 0.8 to 1.1 cm. segment of urethra was excised and replaced by a 1.0 to 1.5 cm. (mean 1.3) acellular urethral matrix tube. The matrix was sutured in place with interrupted 7–0 polyglactin sutures, bridging the urethral defect end-to-end. The wound was closed in layers with a running 7-zero polyglactin suture and the skin was approximated with an inverted running 4-zero polyglactin suture. The feeding tube was fixed to the meatus with 2-zero silk suture and shortened to a visible end of 0.5 cm.

The rabbits received 11.35 mg. enrofloxacin intraoperatively given intravenously and twice daily orally until stent removal at day 7. In the homologous and heterologous groups 4 animals each were sacrificed at 6 months and 3 each were sacrificed at 8 months.

Radiological evaluation. All animals underwent retrograde urethrography under preanesthesia to confirm normal anatomy. A modified 8Fr feeding tube was inserted into the meatus and fixed to the penile skin with a 2-zero silk suture. Urethrography was performed after injecting 1.5 ml. 60% diatrizoate meglumine and x-ray was performed at 2.5 mA. and 56 kV. with a mobile AMX 3 model No. 46–217900G2 x-ray system (General Electric, Milwaukee, Wisconsin). All animals underwent followup retrograde urethrography while supine at 10 days, 3 and 6 weeks, 3 months, and before sacrifice at 6 or 8 months.

Urethral pressure profile. After retrograde urethrography urethral pressure profile was performed twice per animal at sacrifice. The perfusion rate was 0.3 ml. per minute. A multispeed Model 55–2222 pump (Harvard Apparatus, Millis, Massachusetts) was used with a Masterflex speed controlled machine pulling the catheter at a constant rate of 2 cm. per minute. The transurethrally placed 8Fr Argyle feeding tube was connected to a Uniflow transducer (Baxter Healthcare Corporation, Deerfield, Illinois) via a 3-way stopcock with pressure recorded continuously and evaluated using LabVIEW 4.0 software (National Instruments, Austin, Texas) on a Macintosh Quadra 800 Apple Computer (Apple Computer, Inc., Cupertino, California).[6]

Histological evaluation and light microscopy. Pure acellular matrix was prepared for light microscopy by hematoxylin and eosin, and trichrome staining, and for transmission electron microscopy. In so doing the effectiveness of the matrix preparation process was also evaluated. For light microscopy specimens were fixed in 10% buffered formalin for at least 24 hours. After dehydration in graded ethanol solutions the specimens were embedded in paraffin, sectioned at 4 μm., stained with hematoxylin and eosin, trichrome and picrosirius red,[7] and immunostained for α-actin.[8]

For α-actin staining paraffin sections were hydrated to water. After blocking endogenous peroxidase for 20 minutes with 0.3% H_2O_2 in methanol and nonspecific protein binding overnight with 3% normal horse serum in PBS, pH 7.4, containing 0.3% Triton X-100 sections were incubated for 60 minutes at room temperature with mouse monoclonal anti-smooth muscle α-actin (1:6,000) (Sigma Chemical Co.). After washing with buffer sections were immunostained by the avidin-biotin-peroxidase method using the Elite ABC kit (Vector Laboratories, Burlingame, California) with diaminobenzidine plus hydrogen peroxide as the chromagen. Sections were counterstained with hematoxylin. As a negative control, some sections were immunostained without exposure to the primary antibody.[8]

For picrosirius red staining sections were deparaffinized, hydrated and left for 60 minutes in a 0.1% solution of sirius red dissolved in aqueous saturated picric acid. Sections were rapidly washed in running water and dehydrated to xylene.[7] Results were analyzed in accordance with the method of Kiraly et al.[9]

Electron microscopy. Samples were immersion fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M. sodium cacodylate buffer, pH 7.4. After post-fixation in 2% osmium tetroxide the tissue was dehydrated in graded ethanol and propylene oxide, and subsequently embedded in Epon 812. Thick sections (1 mm.) were cut on a microtome, stained with 1% methylene blue and examined with a Leitz Laborlux-S light microscope (Leica Mikroskope und Systeme GmbH, Wetzlar, Germany). Thin sections approximately 900 E were mounted on 200-mesh copper grids and stained with 10% uranyl acetate and lead citrate as contrast agents. Ultrastructural examination was performed with a Model 10 transmission electron microscope (Zeiss, Wetzlar, Germany).

Statistical analysis. Five randomly chosen fields of each

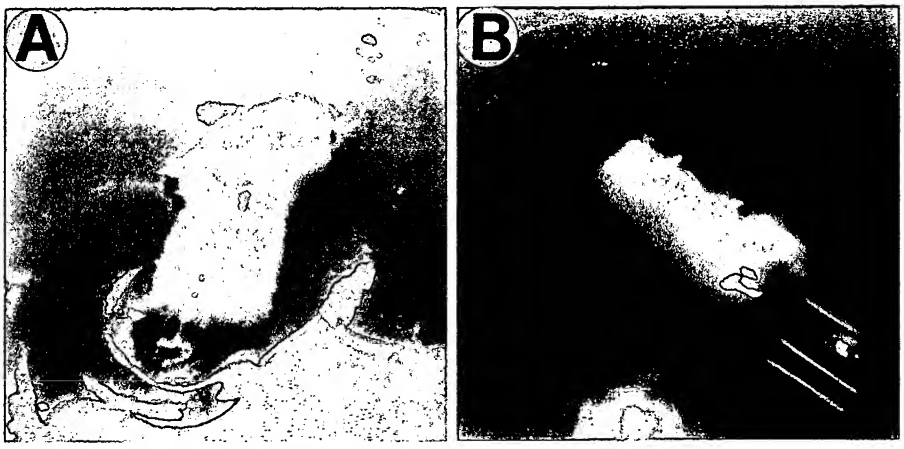

FIG. 1. Acellular matrix before implantation. *A*, rabbit. *B*, dog

specimen stained with picrosirius red were analyzed at ×400 magnification using a DM RB light microscope (Leica Mikroskope und Systeme GmbH) attached to an N 90 digital camera (Nikon, New York, New York). The ratio of collagen types I-to-III was quantified using Adobe PhotoShop, version 4.0 (Adobe Systems, Inc., Mountain View, California) installed on a Power Macintosh G3 (Apple Computer, Inc.). The polarized microscopic pictures were analyzed with custom filters and the mean of these analyzed areas was used to compare group differences with the Mann-Whitney U test (GB-STAT, Dynamic Microsystems, Inc., Silver Spring, Maryland). Values were considered significant at $p < 0.05$ and highly significant at $p < 0.005$.

RESULTS

No animal died during the study and no fistulas were observed. At 8 months retrograde urethrography showed no discernible difference in the matrix implant and host, and there were no significant differences in homologous and heterologous grafts. The borders between the host and matrix were already difficult to differentiate at 6 months (fig. 2). The production process effected graft tissue lengthening by 8% to 10%. However, at sacrifice 8 months postoperatively the implanted tissue had shortened to become closer to its original length.

In this study urethral pressure profiles were obtained in an attempt to determine any subclinical narrowing at the graft level and at anastomotic sites. Results were not notably different in control and matrix implanted animals since in the former we noted a mean of 80 ± 13 cm. water and in the latter with a homologous or heterologous graft the mean was 76 ± 12 cm. water (range 59 to 95) (fig. 3). At regenerate harvest adhesions were noted in groups that were inversely dependent on postoperative time, that is less adhesion with longer postoperative time.

Light and transmission electron microscopy of the pure matrix confirmed the effectiveness of the matrix preparation process. As in our previous studies,[1–5] the acellular grafts appeared as intact frameworks consisting of elastin and collagen fibers. Transmission electron microscopy revealed that the collagen and elastin fibers were thicker in the dog than in the rabbit (fig. 4).[3]

Light microscopy with polarizing lenses of the specimens stained with picrosirius red showed collagen type I as thick, strongly birefringent, yellow or red fibers and collagen type III as thin, weakly birefringent, greenish fibers that were identified as reticulin fibers. When areas of the urethra were analyzed for the ratio of collagen types I-to-III, the procedure for matrix production had caused a notable shift, that is the percent of collagen type III was higher in the species that showed no significant change during the study period (fig. 5). At 6 months the ratio in rabbit matrix was significantly different from that in the normal urethra and it increased only slightly in the ensuing 2 months ($p < 0.05$, see table).

The Mann-Whitney U test demonstrated significant changes in the ratio of canine collagen types I-to-III caused by matrix production ($p < 0.05$). During the 8-month study period the ratio remained almost the same, although changes were still significant compared with the host. Comparing dog matrix to normal rabbit tissue revealed a significantly higher percent of collagen type III than in rabbit urethra (see table and fig. 5).

Light microscopy in control rabbits showed that the U-shaped corpus spongiosum surrounded two-thirds of the circumference of the urethra, which was composed of loose collagen and elastin fibers with numerous outer longitudinally oriented smooth muscle bundles and inner nutritional vessels. On longitudinal section the urethral luminal lining showed prismatic and cuboidal epithelium (fig. 6, *A*). In the heterologous preparation dog urethra was completely surrounded by corpus spongiosum. On longitudinal section smooth muscle was uncommon with a high proportion of collagen covered by prismatic and cuboidal epithelium (fig. 6, *B*).

At 6 months homologous rabbit matrix demonstrated fewer longitudinally oriented smooth muscle cell bundles in the first third of the ingrown matrix than in the normal urethra. In α-actin stained sections irregular areas of ingrowth with few muscle cells were noted, except at the matrix center. However, the number of longitudinal vessels was significantly increased. Smooth muscle increased slightly with additional time for up to 8 months (fig. 7).

In the heterologous implant the surface was completely covered by regular host urothelium and the matrix was streaked with tiny vessels. At 6 months α-actin staining revealed palely stained fibroblasts but no smooth muscle, although at 8 months smooth muscle cells were detected in the matrix anastomosis (fig. 8). The configuration and number of smooth muscle cells were similar to those in normal dog urethra. An increase in α-actin positive cells was evident mainly around the vessels.

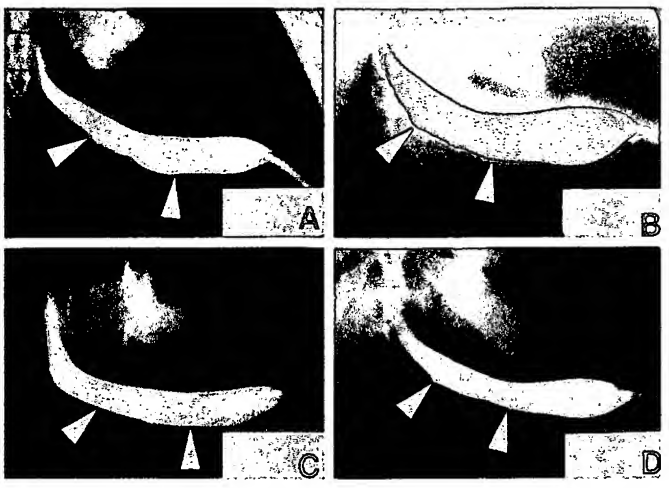

FIG. 2. Retrograde urethrography. *A*, homologous graft at 6 months. Arrowheads indicate graft margins. *B*, homologous graft at 8 months. *C*, heterologous graft at 6 months. *D*, heterologous graft at 8 months.

DISCUSSION

Improved urethral reconstruction depends not only on surgical technique, but also on the material used influences the functional and cosmetic outcome. Previous research has shown that biodegradable materials, such as hyaluronic acid or hydroxyacetic acid polymers, have the best potential for regeneration and function,[10,11] although the degree and quality of urethral regeneration have not been consistent. Data on biodegradable material in various animal models have demonstrated at best the regeneration of urothelium and submucosa but not the complete regeneration of the urethra, including smooth muscle cell formation.[12]

After the encouraging results in our urethral regeneration study with a homologous acellular matrix[5] we expanded our interest to examine partial urethral reconstruction with a heterologous matrix to determine whether the potential pool of successful graft material may be enlarged. Although the process of regeneration was not as rapid or complete as with homologous matrix, the heterologous graft succeeded without inducing antigenicity.

In our previous study we noted increased length caused by matrix production, which we attributed to a loss of contractile tissue. In our current study the relatively greater acontractile component in the dog resulted in a lesser length increase with heterologous matrix production. With time the

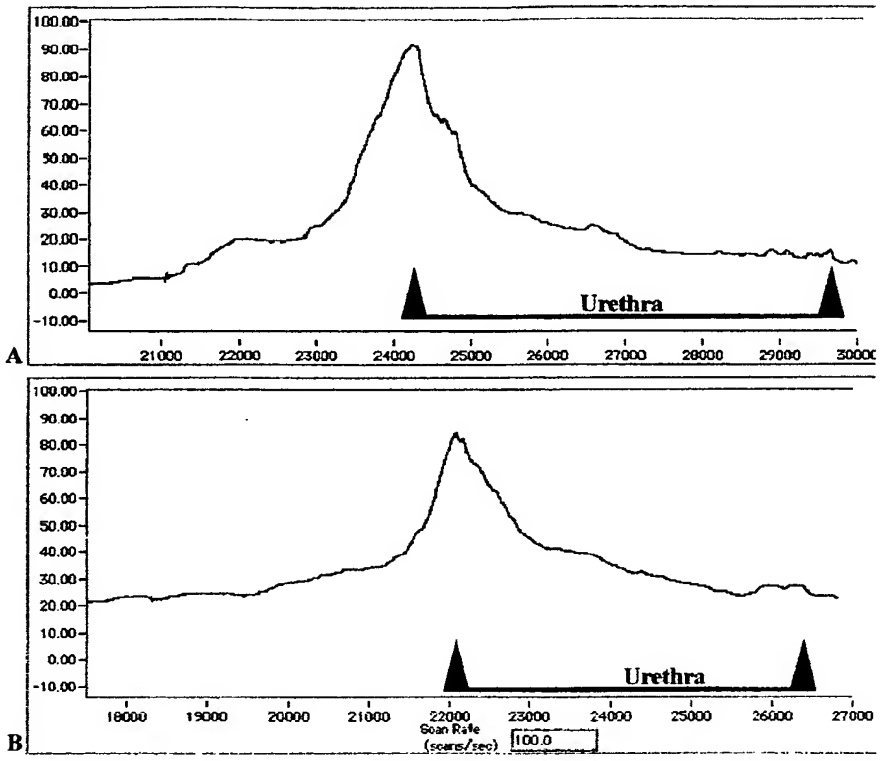

FIG. 3. Urethral pressure profile at 8 months showed no significant difference versus controls. *A*, homologous transplant. *B*, heterologous transplant.

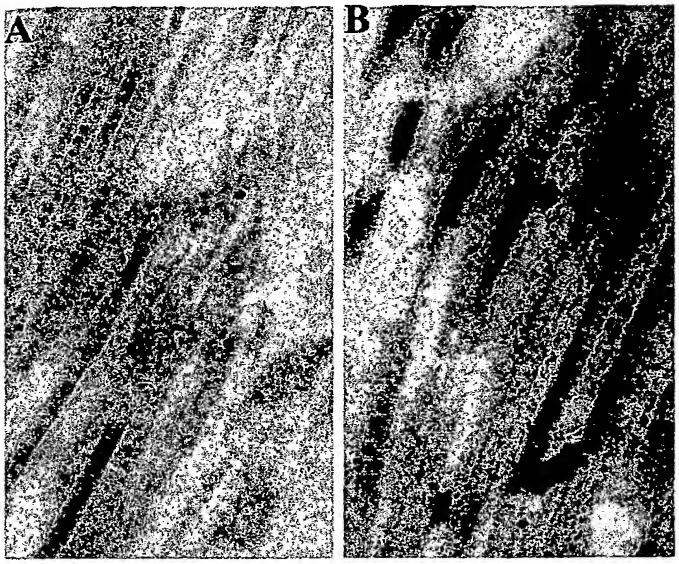

FIG. 4. Transmission electron microscopy shows collagen fibers. *A*, rabbit. *B*, in dog matrix was thicker with apparent connections.

newly ingrown contractile tissue shortened the implant but even after 8 months the heterograft as well as the homograft was still somewhat longer than normal.

Close postoperative care to ensure a consistent period during which the transplant is protected by a stent is essential for reliable histological results.[13] Epithelial keratinization caused by postoperative catheterization seems to be of short duration and was not noted in either group, that is at 6 or 8 months.[11,14] Survival of the experimental animals and absent rejection of the heterologous implant indicate high acceptance of the graft and confirm its acellular nature. Our study showed complete internal epithelialization and angiogenesis with less smooth muscle cell ingrowth into the heterologous than the homologous graft but with no sign of rejection in either implant.

Host urothelium covered the surface of the heterologous transplant but the surface was not like that in the host or homologous transplant. Neovascularization increased with time with advancement from matrix edges to the center. These smaller changes were related to postoperative time since the heterologous graft required a longer interval to acquire the same histological appearance as the homologous graft.

After 6 months myofibroblasts were still present in the heterologous graft, as opposed to our previous observation in the homologous implant, in which they were not detected after 6 weeks.[5] Although myofibroblasts change into smooth muscle cells, it is still not known what causes this transformation.[15,16] The high number of myofibroblasts in the graft at up to 6 weeks was not equivalent to the number of smooth muscle cells later. This finding may have been due to a persistently high amount of transforming growth factor-α for 8 months, as previously reported,[5] which inhibited smooth muscle migration. Heparin binding epidermal growth factor is only up-regulated from postoperative day 10 until month 3,[5] which may explain the small number of smooth muscle cells. Compared with previous results in bladder matrix regeneration of the urethral matrix requires more time and this difference may be affected by the different functions of these 2 organs.

Myofibroblasts migrated into the graft before the first vessels were observed and smooth muscle cells were later noted in areas with no continuous connection to ingrowing smooth muscle cells. In a study of Wu et al smooth muscle cells in the acellular matrix were found to be histochemically identical to those in normal fetal bladder.[16] Their data did not reveal the growth of smooth muscle cells across the acellular matrix, only their development.

In our study smooth muscle bundles were observed to grow into a third of the homologous matrix scaffold by 6 months

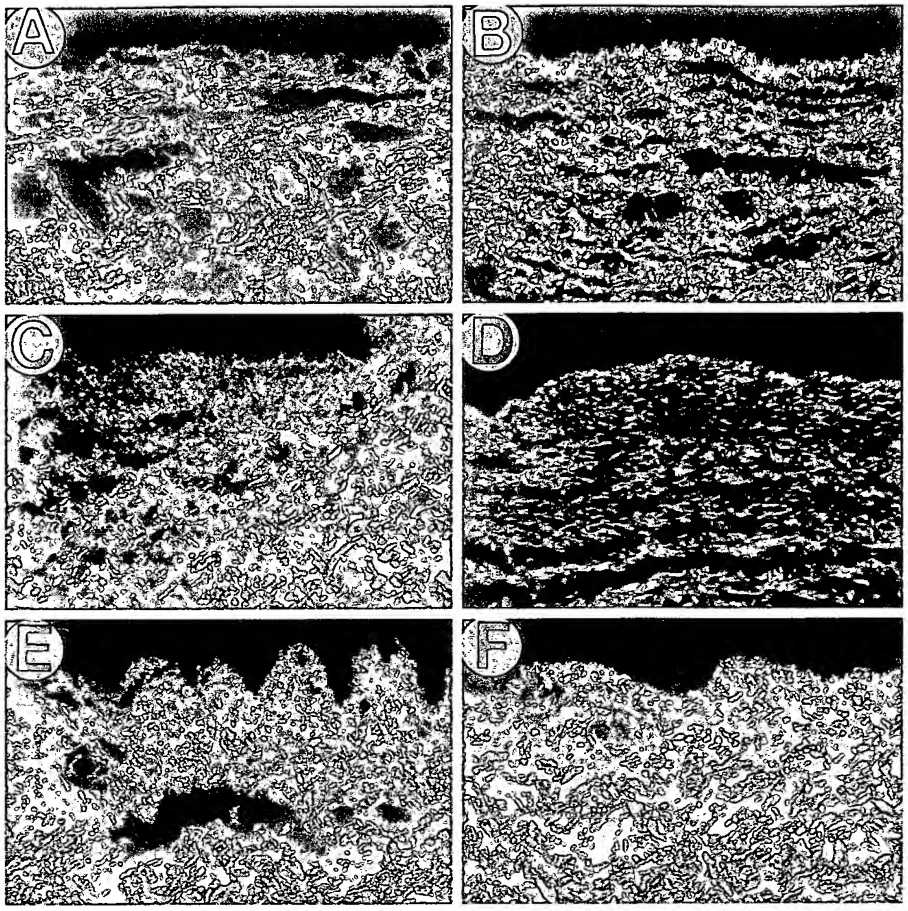

FIG. 5. Light microscopy at 8 months. *A*, normal rabbit urethral tissue. *B*, normal dog urethral tissue. *C*, processed rabbit matrix. *D*, dog matrix. *E*, implanted rabbit graft. *F*, implanted dog graft. Picrosirious red stain, reduced from ×40.

Collagen types I and III in normal urethral tissue, matrix preparation and implant

	Rabbit*		Dog†	
	Mean % Collagen I ± SD	Mean % Collagen III ± SD	Mean % Collagen I ± SD	Mean % Collagen III ± SD
Normal urethra	84.6 ± 1.2	15.4 ± 1.2	92.3 ± 1.0	7.7 ± 1.0
Matrix	72.7 ± 3.0	27.3 ± 3.0	79.3 ± 2.4	20.7 ± 2.4
Implant (mos.):				
6	72.4 ± 2.7	27.6 ± 2.7	79.1 ± 0.6	20.9 ± 0.6
8	72.7 ± 1.2	27.3 ± 1.2	78.9 ± 1.5	21.1 ± 1.5

* Collagen I:III ratio was significantly different from normal at 6 ($p = 0.037$) and 8 ($p = 0.002$) months.
† Collagen I:III ratio was significantly different from normal at preparation ($p = 0.014$) and remained so for more than 8 months ($p = 0.003$).

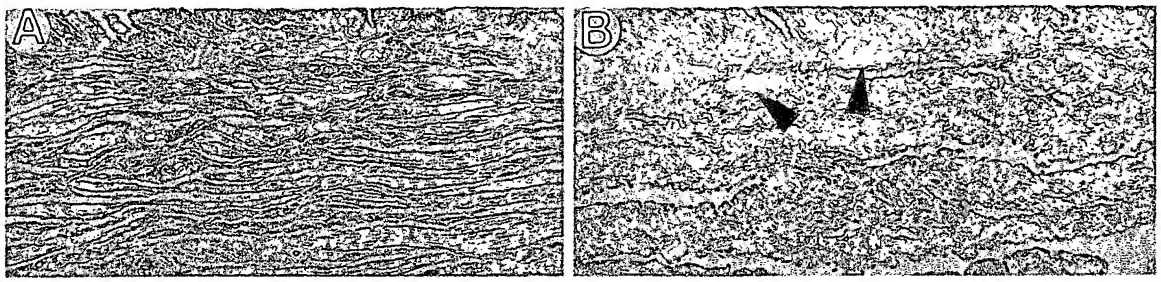

FIG. 6. Urethra. *A*, many smooth muscle cells in normal rabbit urethra. *B*, single smooth muscle cells (arrowheads) in dog urethra. Trichrome stain, reduced from ×20.

and, although longitudinal order was poor, there was a slight improvement after an additional 2 months. In the heterologous transplants only disseminated myofibroblasts were observed in the matrix after 6 months and the first smooth muscle cell ingrowth was first observed at 8 months. The orientation in the scaffold appeared similar to that in the canine donor tissue. Electron microscopy demonstrated differences in the dog and rabbit scaffolds, which may explain the slower smooth muscle cell migration and development. The number of myofibroblasts in the homologous versus the heterologous graft may explain the small number of smooth muscle cells in the latter after 8 months. In addition, the

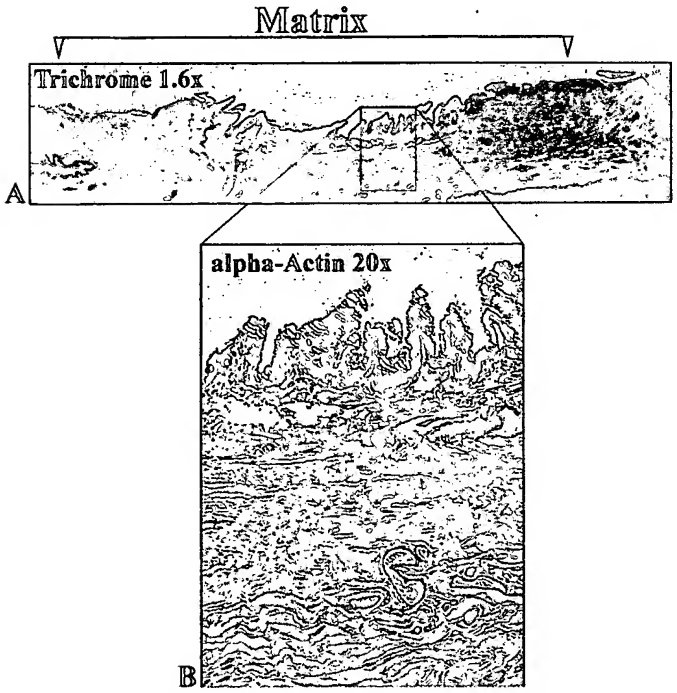

FIG. 7. Longitudinal section of rabbit graft at 8 months demonstrates regular urothelium over whole tissue surface. Trichrome stain, reduced from ×1.6. Inset shows smooth muscle cells close to center of graft. α-Actin stain, reduced from ×20.

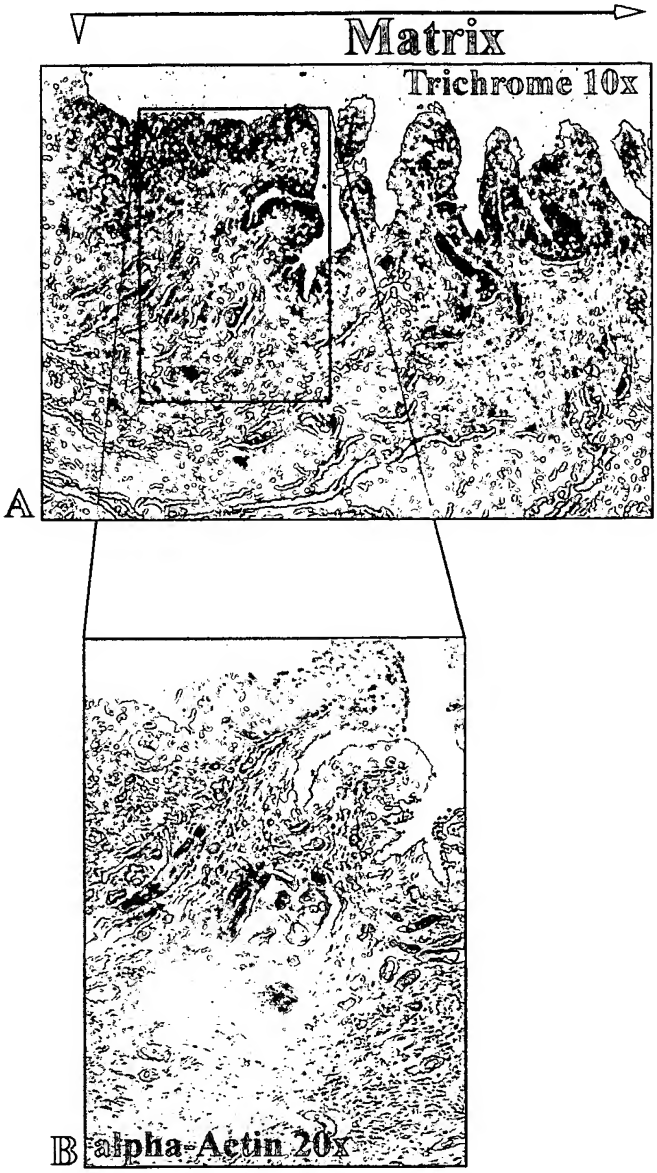

FIG. 8. At 8 months first smooth muscle cells were noted in dog graft. Urothelium was similar to that in host. Trichrome stain, reduced from ×10. Inset shows α-actin stain, reduced from ×20.

amount of myofibroblasts may depend on the scaffold structure.

Assuming that the acellular matrix carries no genetic information to influence regeneration, the scaffold appears to influence the regeneration process. In a bladder regeneration study Piechota et al analyzed several species and found scaffold differences on transmission electron microscopy in 2 species.[3] In our study collagen bundles in the dog matrix appeared thicker than in the rabbit matrix and were connected to each other rather than individualized. Baskin et al reported that normal urethral spongiosum and urethral stricture scar tissue were principally collagen types I and type III, respectively.[17] They postulated that normal fibroblasts synthesize each type but a change in the surrounding environment causes collagen synthesis to become imbalanced, resulting in urethral stricture. This finding was demonstrated by increased messenger RNA for collagen type I in scar tissue. We used picrosirius red polarization to reveal collagen ratio changes,[18] a technique that has been used successfully in other organs. To the method of Kiraly et al[9] we added a digital camera and PhotoShop, version 4.0 installed on a custom Macintosh G3 computer for further objectivity.

To our knowledge the observation that the process of matrix production appears to cause a change in the ratio of collagen types I-to-III that may be species dependent with greater alteration in the dog has not been previously evaluated. However, it remains unclear how and why the ratio in the implanted scaffold changed only partially postoperatively and seemed to be more or less the same after 8 months, although the decreased ratio became significant even in the homologous graft compared with donor tissue. Because scar development has been explained as a result of increased collagen type I and decreased collagen type III production by fibroblasts,[17] a decrease in the ratio of collagen types I-to-III may explain the lack of stricture development in our specimens. It is still unclear how the surrounding environment regulated the amount of each collagen type synthesized by fibroblasts since the implanted graft did not cause a significant inflammatory reaction. The altered collagen ratio in the acellular matrix and the appearance of the graft at 8 months do not represent an early stage of wound healing, in which collagen type III may be described as functioning with more flexibility or distensibility.[19] However, retrograde urethrography and the urethral pressure profile revealed no difference from normal urethra at 6 months.

CONCLUSION

The heterologous and homologous transplants demonstrated normal functional regeneration, although the homologous graft showed earlier histological host equivalence. There was no evidence of tissue rejection of the heterologous acellular matrix.

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